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13. ABSTRACT ***(Maximum 200 Words)***

The bioavailability of IGF-II, a molecule implicated in breast tumorigenesis, was recently shown by our laboratory to be modulated by TIMP-1 through controlling MMP-mediated IGFBP degradation. To investigate the exact role of IGF-II in breast tumorigenesis and examine IGF-II-mediated events potentially regulated by TIMP-1 expression we generated independent MMTV-IGF-II transgenic mice. Since increased proliferation and/or decreased apoptosis precede tumor development, we investigated the effects of IGF-II overexpression on proliferation during mammary development and its effect on apoptosis during postlactation involution. We found that elevated levels of IGF-II inhibitied mammary epithelial proliferation in vivo. The delay in ductal morphogenesis correlated with an elevation of the phosphatase PTEN, in the transgenic tissue. Higher PTEN levels resulted in reduced phosphorylation of Akt/PKB and a subsequent reduction in cylcin D1 protein levels. We have also observed a delay in ductal morphogenesis when the oncogene, erbB2 is overexpressed. IGF-II overexpression also affected epithelial apoptosis in vivo. Specifically, high levels of IGF-II resulted in delayed postlaction involution through inhibiting mammary epithelial apoptosis. This inhibition of apoptosis was associated with a sustained phosphorylation of Akt/PKB in the transgenic tissue. These data demonstrate that an elevation IGF-II can affect the pre-tumorigenic events, events potentially controllable through manipulating TIMP-1.

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Roger Moorhead — July 25, 2000

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Introduction

Among growth factors implicated in breast cancer, insulin-like growth factors (IGFs), especially IGF-II, are gaining increasing recognition as important mitogens. Our recent finding that tissue inhibitor of metalloproteinase (TIMP)-1 can modulate IGF-II bioavailability suggests that TIMP-1 may regulate processes other than extracellular matrix degradation during breast cancer progression. To further investigate the role of IGF-II in breast tumorigenesis and its modulation by TIMP-1, we generated transgenic mice in which both IGF-II and Timp-1 have been genetically manipulated. **We postulate that overexpression of IGF-II will promote mammary epithelial proliferation eventually resulting in the formation of mammary tumors. In addition the levels of free IGF-II capable of stimulating epithelial proliferation can be regulated by TIMP-1.** The overall strategy of this proposal is to generate MMTV-IGF-II transgenic mice that should develop mammary tumors and genetically combine these mice with transgenic mice either overexpressing TIMP-1 (TIMP-1^{high}) or express TIMP-1 antisense RNA (TIMP-1^{low}). Effects of TIMP-1 dysregulation on IGF-II-mediated morphological, cellular and molecular events in mammary tumorigenesis will be investigated. Four specific aims were identified; (1) Generate transgenic mice expressing IGF-II in the mammary tissue, (2) Confirm mammary IGF-II expression, (3) Analyze the course of mammary tumorigenesis, and (4) Establish and characterize double transgenics. To date, we have successfully generated MMTV-IGF-II transgenic mice and characterization of these mice have resulted in the acceptance of one manuscript publication describing the effect of IGF-II overexpression on postlactation involution (appendix 2) and the preparation of another manuscript describing the effect of IGF-II overexpression on pubertal mammary ductal morphogenesis (appendix 1). The highlights of these manuscripts are presented below and the full manuscripts are provided as appendices.

Generation and Characterization of MMTV-IGF-II Transgenic Mice

To derive transgenic mice overexpressing IGF-II, an expression construct containing a human IGF-II (hIGF-II) cDNA under the control of the MMTV-LTR (Fig. 1A, appendix 2) was microinjected into one-cell zygotes. Founder animals were identified by probing Southern blots of *Pst*I cleaved tail DNA with a hIGF-II DNA probe which detected an 833 bp transgene-specific fragment, as well as a 1391 bp endogenous IGF-II fragment (Fig. 1B, appendix 2). Three male founders were identified (MI1, MI12 and MI16) and an independent transgenic line established from each founder mouse.

Next we screened for IGF-II expression in the mammary tissue. Mammary mRNA from 35 day-old wild type and transgenic mice was analyzed by RT-PCR using both transgene-specific and control primer sets. A forward primer located in the transcribed portion of the MMTV promoter and reverse primer in the IGF-II transgene (primers 1 and 3, Fig. 1A, appendix 2) produced 600-800 bp fragments that were evident only in the mammary tissue of transgenic mice (Fig. 1C, appendix 2). The 296 bp fragment generated from the interleukin-2 control primers was visible in every lane and served as a positive control for individual PCR reactions. In addition, in situ hybridization using a digoxygenin-labeled IGF-II antisense riboprobe demonstrated that mammary epithelial cells in transgenic tissue expressed IGF-II and the level of expression was considerably higher compared to wild type controls (Fig. 1A-D, appendix 1 and Fig. 1D and E, appendix 2). Therefore, we have generated transgenic mice that overexpress IGF-II in the epithelial cells of mammary tissue. During the characterization of these mice we observed a delay in ductal morphogenesis and a delay in postlactation mammary involution.

Overexpression of IGF-II Delays Mammary Ductal Morphogenesis

Mammary development begins during embryogenesis and continues postnatally until around 10-12 weeks of age. In response to ovarian and pituitary hormones, terminal end buds (TEBs) form at the tips of the epithelial ducts and ductal lengthening ensues (1). At day 15 of age a rudimentary ductal tree exists and these epithelial ducts extend into the empty mammary fat pad reaching the lymph node around 35 days of age. Lengthening of the ducts continues until the end of the mammary gland has been reached at around 75 days of age in FVB mice. When the ducts reach the end of the mammary fat pad the TEBs typically disappear (1). A number of factors in addition to estrogen have been shown to participate in this developmental process including growth hormone (2-4). More recent studies have demonstrated that growth hormone does not directly affect mammary morphogenesis rather it stimulates the production of IGF-I. However, the role of IGF-II in this process is poorly understood. Therefore, we examined the effect of elevated IGF-II on ductal morphogenesis.

Although IGF-II is a potent mitogen for mammary epithelial cells and breast cancer cell lines in culture (5-10), we demonstrate here that IGF-II overexpression limits ductal morphogenesis by inhibiting epithelial proliferation. At the whole mount level we found that the length of the mammary epithelial ducts were significantly shorter in the MMTV-IGF-II transgenic mice compared to wild type controls (Fig. 2 A-C, appendix 1) at day 55 of development. The epithelial ducts of wild type mice had grown on average 49.1 ± 3.0 mm beyond the lymph node while epithelial ducts of MI1 and MI12 transgenic lines had extended 35.4 ± 4.1 mm ($p < 0.05$) and 35.8 ± 0.7 mm ($p < 0.05$), respectively.

This decrease in ductal length was also observed when pellets containing recombinant IGF-II were implanted into the mammary glands of wild type mice. In addition, there was also a significant reduction in the number of epithelial ducts in the MMTV-IGF-II mice (12.5 ± 1.0, $p < 0.05$ in MI1, 11.5 ± 1.3, $p < 0.05$ in MI12 and 16.2 ± 0.9 in wild type mice).

To investigate whether these differences in epithelial branching arose during a particular period of mammary development, we also examined mice at days 15, 35 and 75. At days 15 and 35, no significant differences were observed in the length of the epithelial ducts. At day 75 we observed that the MMTV-IGF-II mice have significantly more TEBs remaining in the fourth inguinal mammary gland compared to wild type controls (8.5 ± 1.6 for MI1 vs 2.4 ± 0.9 for Wt, $p < 0.05$ and Fig. 3B vs Fig. 3A, appendix 1). Since TEBs generally disappear once ductal lengthening has finished, these results suggest that ductal lengthening is nearly complete in the wild type mice but still occurring in a substantial number of ducts in the MMTV-IGF-II transgenic mice.

Extension of the epithelial ducts into the empty mammary fat pad is dependent on epithelial proliferation and thus a decrease in proliferation may account for the delay in ductal morphogenesis. At day 55 of development the percentage of mammary epithelial cells proliferating was significantly reduced in the transgenic tissue (6.7 ± 1.0 % for Wt vs 3.8 ± 0.7 % for Tg, $p < 0.05$ and Fig. 3, appendix 1). Since the decrease in epithelial proliferation and ductal lengthening could not be attributed to alterations in the circulating hormone levels or gelatinase activity, several signal transduction pathways downstream of the type I IGF receptor were investigated. We found that Erk1/Erk2, p38 MAPK, and ATF-2 (target of JNK/SAPK) were not altered in the transgenic tissue (Fig. 4A-C, appendix 1). We did however, identify that the levels of phosphorylated Akt/PKB were significantly reduced in the MMTV-IGF-II mice (Fig. 4D and 4H, appendix 1). Akt/PKB phosphorylation is regulated by PI-3 kinase that is also downstream of the IGF-IR. Akt/PKB is primarily perceived as an antiapoptotic molecule but more recent studies have indicated that Akt/PKB can modulate cell cycle progression through regulation of cyclin D1 stability. When the cyclin D1 levels were examined, we found that the transgenic tissue had lower levels of cyclin D1 (Fig. 4E and 4I, appendix 1) suggesting that the reduced epithelial proliferation in the transgenic mammary tissue was mediated, at least in part, by decreased Akt/PKB phosphorylation and subsequent reductions in cyclin D1 protein levels.

It was unclear exactly how IGF-II overexpression resulted in reduced Akt/PKB phosphorylation when activation of the IGF-IR by IGF-II should lead to a sequential increase in IRS-1, PI-3 kinase and Akt/PKB activation until the levels of PTEN were examined. PTEN is a phosphatase that counteracts the actions of PI-3 kinase-mediated activation of Akt/PKB. Western analysis demonstrated a significant elevation in the levels of PTEN in MMTV-IGF-II mice (Fig. 4F, 4G and 4J, appendix 1). These results suggest that IGF-II overexpression, either directly or indirectly, augments PTEN levels resulting in a reduction of phosphorylation Akt/PKB and enhanced cyclin D1 degradation subsequently leading to a delay in ductal morphogenesis.

Overexpression of IGF-II Delays Postlactation Mammary Involution

A potential mechanism by which IGF-II can promote mammary tumorigenesis is through inhibiting apoptosis. A large body of in vitro and in vivo evidence demonstrates

that IGF-I inhibits apoptosis in several cell types (11-17). On the other hand, the antiapoptotic properties of IGF-II have been shown in vitro, but remain to be proven in vivo. The intracellular molecules responsible for mediating cell survival downstream of the IGF-IR have also not been characterized in vivo. We asked the question whether and how IGF-II overexpression exerts antiapoptotic effects in vivo.

To address this, the event of postlactation mammary involution was selected as a model for our studies. Extensive mammary epithelial proliferation and differentiation culminates in the generation of lobulo-alveoli for lactation. However, these structures undergo scheduled regression following the loss of suckling stimuli, accumulation of milk, and decrease in lactogenic hormones. The lobulo-alveolar collapse is a result of massive mammary epithelial apoptosis (18;19). These events can be synchronized by removal of the litter at a specific day of lactation and have been widely studied at the morphological, cellular and molecular levels (18;20-25). Therefore, postlactation mammary involution offered an appropriate model to uncover the effects of IGF-II on cellular apoptosis and to elucidate the underlying molecular events in a physiological environment.

Overexpression of IGF-II was achieved in the mammary epithelium during involution of MMTV-IGF-II mice (Fig. 1D-G and Fig. 2A, appendix 2). Very low levels of endogenous IGF-II mRNA were evident in mammary tissue from wild type mice and these levels were maximal on days 10 of lactation and 1 of involution. On the other hand, transgenic IGF-II mRNA was observed throughout involution, and its expression was 50-100 fold that of the endogenous IGF-II mRNA levels.

As an initial measure of the gross mammary alterations that ensue during involution, the weight of 4^{th}-inguinal mammary fat pads relative to the body weight of the mouse, were monitored. An initial increase in this value was expected after pup removal due to milk accumulation, and was observed in both wild type and transgenic mice at d1i. As involution proceeds, this ratio declines in a characteristic manner leveling out around d4i, a time period of maximal epithelial apoptosis. We found that the mammary gland-to-body weight ratio was consistently higher from d1i to d4i in the MMTV-IGF-II mice compared to wild type controls and these differences were significant at several of the time points (Fig. 3A, appendix 2).

To ascertain whether elevated expression of IGF-II resulted in morphological alterations in the epithelial ductal structures, whole mount analyses were conducted on each day beginning at 10L until d8i. At day 10L, the mammary gland is packed with large, milk-filled lobulo-alveoli. Progressive, scheduled involution then leads to lobulo-alveolar regression that is followed by the clearing of mammary epithelial cellularity and the reconstitution of adipose tissue. We found that the process of mammary involution was delayed in IGF-II overexpressing mice. Specifically, the bud-like, lobulo-alveolar structures persisted for a longer period before regression in MI1 transgenics and these differences were greatest at d4i (Fig. 3B,C, appendix 2). We also examined the number of structurally intact lobulo-alveoli remaining on specific days of involution. Lobulo-alveolar collapse was clearly delayed in MI1 transgenic mammary glands at d4i (Fig. 3D,E, appendix 2) and a higher number of lobulo-alveoli were present from d3i to d6i in the mammary glands of transgenic mice than of controls (Fig. 3H, appendix 2).

To determine the basis of delayed lobulo-alveolar collapse in the transgenic tissue, we elucidated the apoptotic index of mammary epithelial cells throughout

involution. Involution in wild type mice showed that apoptotic cells were first detected at d1i and peaked at d4i while the percentage of total mammary epithelial apoptosis in MMTV-IGF-II mice lagged behind that of the controls. Further, when intact lobulo-alveoli were considered, the number of apoptotic cells was approximately half of that observed in the control tissue, from d3i to d5i ($p < 0.05$, Fig. 4C, appendix 2). Epithelial apoptosis could also be inhibited by implantation of recombinant IGF-II pellets into involuting mammary glands of wild type mice (Fig. 6, appendix 2) providing further evidence that IGF-II protein inhibited local epithelial apoptosis.

We further explored the molecular basis of the antiapoptotic effect of IGF-II. A number of in vitro studies have shown that IGF-I and IGF-II inhibit apoptosis through the activation of Akt/PKB (26-29). We investigated whether reduced mammary epithelial apoptosis in transgenic tissue was linked to activation of Akt/PKB. Phosphorylated Akt/PKB was detected at 10L in both wild type and transgenic mice and its levels increased approximately 5-fold at d1i (Fig. 5A,B, appendix 2). In wild type mice, the levels of p-Akt/PKB diminished rapidly by d2i and were undetectable thereafter. It was striking that the levels of phosphorylated Akt/PKB remained elevated until d3i and were observed as late as d4i in the MMTV-IGF-II mice.

Akt/PKB phosphorylation is regulated by the phosphorylation status of the lipid phosphatidylinositol (3,4,5)-triphosphate (PIP-3). Since the phosphatase PTEN can dephosphorylate PIP-3, levels of PTEN can limit the duration of Akt/PKB phosphorylation (30-32). There were no differences in the levels of PTEN protein in the wild type versus the transgenic mammary tissue (Fig. 5C, appendix 2). Interestingly, it appeared that the levels of PTEN protein began to increase at d3i, the time the Akt/PKB phosphorylation was lost.

Although the downstream targets of Akt/PKB are still poorly understood it has been demonstrated that Akt/PKB can phosphorylate Bad in tissue culture systems (reviewed in (33-35)). We attempted to assess the phosphorylation status of Bad in our mammary tissue. The phosphorylation-specific antibody (Ser112) was unable to detect phosphorylated Bad during the events of physiological apoptosis. Activation of Erk1 and Erk2 has also been implicated in inhibiting apoptosis, possibly through phosphorylation of Bad (36;37). Since IGF-II-mediated signaling through the IGF-IR can stimulate the Erk pathway (38) we examined the levels of phosphorylated Erk1 and Erk2 to seek the involvement of this pathway in the antiapoptotic response of IGF-II. There were no differences in Erk1 or Erk2 in the wild type and transgenic involuting mammary tissue (Fig. 5D, appendix 2). Together, this data revealed Akt/PKB as the predominant molecule in the antiapoptotic signaling of IGF-II in vivo.

The long-term biological consequence of delayed mammary involution was revealed in two multiparous transgenic mice that we have examined to date. An MI16 female that had undergone three pregnancies displayed a focal abnormal area in the 4^{th}-inguinal mammary fat pad and an MI1 double hemizygous female showed a similar abnormality in both of its 4^{th}-inguinal mammary glands after two pregnancies. Initial whole mount analysis of the mammary tissue (Fig. 1A, appendix 3) identified areas that resembled hyperplastic alveolar nodules (HANs) (39;40). Subsequent histological analyses revealed regions of irregular ductal cystic dilatation (Fig. 1B,C, appendix 3) and epithelial cell proliferation (Fig. 1D, appendix 3). In situ hybridization with an IGF-II-specific riboprobe confirmed that the epithelial cells in these abnormal areas indeed

expressed IGF-II (Fig. 1E, appendix 3). Such atypical areas were not found in multiparous wild type control females having undergone similar numbers of pregnancies. These abnormalities demonstrate incomplete mammary involution, chronic mastitis and focal epithelial hyperplasia in the MMTV-IGF-II mice.

Key Research Accomplishments

- Generated MMTV-IGF-II transgenic mice and identified 3 founder animals
- Created PCR screening strategy to genotype subsequent offspring
- Confirmed IGF-II transgene expression in mammary tissue by RT-PCR and In situ hybridization
- Demonstrated that IGF-II expression was elevated 50-100-fold in transgenic mammary tissue compared to wild type mammary tissue.
- Observed a delay in postlactation mammary involution in MMTV-IGF-II transgenic mice characterized by a maintenance of mammary weight and lobulo-alveoli
- Demonstrated a decrease in epithelial apoptosis during mammary involution and showed that this delay was mediated through sustained phosphorylation of Akt/PKB
- Found areas of focal epithelial hyperplasia in multiparous transgenic females
- Observed a delay in mammary ductal morphogenesis in MMTV-IGF-II transgenic mice as characterized by a significant decrease in the number and length of the epithelial ducts
- Demonstrated that the decrease in ductal morphogenesis was mediated by a significant reduction in mammary epithelial proliferation.
- Showed that a decrease in the levels of phosphorylated Akt/PKB with a subsequent decrease in cyclin D1 protein levels could account for the diminished epithelial proliferation
- Showed that reduced phosphorylated Akt/PKB in the MMTV-IGF-II tissue was a result of increased PTEN protein levels
- Found that IGF-II overexpression did not affect mammary physiology during pregnancy.

Reportable Outcomes

Moorehead, R.A., Fata, J.E., and Khokha, R. Overexpression of IGF-II inhibits mammary epithelial proliferation and pubertal ductal morphogenesis in MMTV-IGF-II transgenic mice. (manuscript in preparation, appendix 1)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Inhibition of mammary epithelial apoptosis through sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice. Cell Death and Differentiation (in press, appendix 2)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Inhibition of Mammary Epithelial Apoptosis Through Sustained Phosphorylation of Akt/PKB in MMTV-IGF-II Transgenic Mice. Society of Reproductive Biology, 2000. (presentation)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Delay of mammary involution and epithelial apoptosis in vivo by IGF-II. 5th International Symposium on Insulin Like Growth Factors, 1999. (abstract).

Conclusions

Generation and characterization of MMTV-IGF-II transgenic mice has revealed that overexpression of IGF-II affects both mammary epithelial proliferation and apoptosis in vivo. Both of these events regulated by IGF-II appear to involve the levels of phosphorylated Akt/PKB. During mammary development elevated IGF-II leads to a decrease in phosphorylated Akt/PKB apparently through elevating the levels of the phosphatase, PTEN. The antiapoptotic effects of IGF-II were correlated with sustained phosphorylation of Akt/PKB eventually resulting in focal hyperplasia in multiparous transgenic females. These results are interesting in light of the fact that elevated Akt/PKB activity and loss of PTEN activity are frequently observed in several tumor types. Now that the effects of IGF-II overexpression on mammary physiology have been established and downstream effector molecules identified, we can proceed with the generation of double transgenic mice containing elevated IGF-II expression with alterations in mammary Timp-1 levels to assess the ability of TIMP-1 to modulate IGF-II bioactivity. In parallel we will continue to monitor the MMTV-IGF-II transgenic mice for the development of mammary tumors.

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Overexpression of IGF-II inhibits mammary epithelial proliferation and pubertal ductal morphogenesis in MMTV-IGF-II transgenic mice.

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Abstract

Until recently, insulin-like growth factor (IGF)–II was considered primarily a fetal mitogen, but it is now known that it is expressed in some adult tissues including the mammary gland. Although IGF-II is a potent mitogen for mammary epithelial cells and breast cancer cell lines in culture, we demonstrate here that IGF-II overexpression limits ductal morphogenesis by inhibiting epithelial proliferation. We found that the length of the mammary epithelial ducts were significantly shorter in the MMTV-IGF-II transgenic mice compared to wild type controls, a phenotype that was confirmed through implantation of IGF-II-containing pellets into the mammary glands of wild type mice. Moreover, there was also a significant reduction in the number of epithelial ducts in the MMTV-IGF-II mice. The growth retardation phenotype was apparent until day 75 of development as a significant number of TEBs were still visible in the transgenic mammary tissue. Epithelial proliferation was significantly lower (1.8-fold) within the ducts and TEBs of the transgenic tissue. The decrease in epithelial proliferation and ductal lengthening could not be attributed to alterations in the circulating 17-β estradiol or progesterone levels or gelatinase activity in the mammary tissue. We further investigated the signal transduction pathways downstream of the type I IGF receptor and found that Erk1/Erk2, p38 MAPK, or ATF-2 (target of JNK/SAPK) were not altered in the transgenic tissue. We identified that the levels of phosphorylated Akt/PKB were significantly reduced in the MMTV-IGF-II mice. We also found that the transgenic tissue had lower levels of cyclin D1, a key regulator of cell cycle progression and a protein that is regulated by Akt/PKB. The lower levels of phosphorylated Akt/PKB were not a result of decreased IRS-1-mediated activation of Akt/PKB but could be explained by an increase in the level of the Akt/PKB inhibitor, PTEN, in MMTV-IGF-II mice. These results suggest IGF-II overexpression during physiological mammary development inhibits epithelial proliferation and ductal lengthening through an elevation in PTEN levels with subsequent suppression of Akt/PKB phosphorylation and cyclin D1 protein levels.

Introduction

Mammary ductal morphogenesis starts during embryogenesis as a rudimentary ductal tree emanating from a single primary duct (reviewed in (1)). It is thought that this mammary development results from circulating maternal hormones and following birth a decrease in these hormones causes a reduction in terminal end bud (TEB) size and slows ductal development. Around 3-4 weeks of age an increase in ovarian estrogen secretion induces the reappearance of TEBs and increases the rate of ductal elongation (1). A number of factors in addition to estrogen have been demonstrated to participate in this developmental process such as progesterone (2-4), growth hormone (3-5), prolactin (3,4,6,7), epidermal growth factor (8-10), fibroblast growth factor (11) and transforming growth factor-β (12,13). Lengthening of the mammary ducts continues until the end of the fat pad is reached at which time the TEBs disappear and lengthening ceases (1).

More recent studies have demonstrated that growth hormone does not directly effect mammary morphogenesis rather it stimulates the production of insulin-like growth factor-I (IGF-I), presumably by the stromal compartment. IGFs have potent mitogenic effects on various cell types in vitro (reviewed in (14-21)) and have been shown to promote mammary epithelial proliferation and proliferation of breast cancer cell lines (22-27). IGFs elicit their mitogenic effects by binding to the IGF-I receptor (IGF-IR) on the surface of mammary epithelial cells and inducing signal transduction through this tyrosine kinase receptor. In vivo, IGF-I can by itself, promote ductal lengthening and act synergistically with estrogen during mammary development. The requirement of IGF-I for normal ductal development has been demonstrated in IGF-I knockout mice where it was shown that mammary development is significantly reduced in these mice as measured by the number of TEBs and the percentage of the mammary gland occupied by epithelial ducts (28). Further, estrogen was incapable of stimulating mammary development in the absence of IGF-I thus demonstrating the importance of IGF-I during this process.

Based on the potent mitogenic effect of IGF-I and IGF-II on mammary epithelial cells in vitro, elevated levels of IGF-I or IGF-II should augment mammary epithelial proliferation in vivo. In fact, transgenic overexpression of IGF-I in mammary tissue using the mouse mammary tumor virus (MMTV) promoter resulted in precocious lobulo-alveolar development although ductal lengthening or branching was not examined (29). Other mammary-specific IGF transgenics have been generated using the whey acidic promoter (WAP) (30-32) and sheep β-lactoglobulin promoter (33) to drive transgene expression. These promoters are only expressed in the mammary gland at high levels during pregnancy and lactation thus limiting their usefulness as developmental tools. Until a recent study by Riechert and Wood (34) showed that IGF-II is expressed in mammary tissue during pubertal development and gestation the effect of IGF-II on mammary morphogenesis had been overlooked since it was generally accepted that IGF-II expression becomes silenced in most adult tissues due to imprinting of the gene (35,36). We have extended the observation by Riechert and Wood and shown that IGF-II is also expressed in adult mammary tissue during postlactation involution (37). These observations suggest that IGF-II has the potential to regulate mammary morphogenesis.

Since the in vivo effects of elevated IGF-II on mammary epithelial proliferation have never been investigated we examined postnatal mammary ductal morphogenesis in transgenic mice in which IGF-II expression was directed using the MMTV promoter. We found that overexpression of IGF-II inhibited, rather than promoted, mammary epithelial proliferation and ductal lengthening during pubertal development. This anti-proliferative effect of IGF-II involved decreased phosphorylation of Akt/PKB as well as a reduction in cyclin D1 protein levels and Rb phosphorylation. The decrease in Akt/PKB phosphorylation appeared to be mediated by an elevation in PTEN protein, rather than reduction in IRS-1 activation in the MMTV-IGF-II transgenic mammary tissue. These results indicate that IGF-II overexpression inhibits mammary morphogenesis in vivo and suggest that the mitogenic properties of growth factors elicited in vitro are not conserved under all physiological conditions in vivo.

Material and Methods

Whole Mount Analysis. The 4th-inguinal mammary glands were removed from mice and placed on glass slides. After air-drying for 10 min, the mammary tissue was fixed overnight in Clarke's solution (75% ethanol, 25% glacial acetic acid). Fixed mammary glands were dehydrated in 70% ethanol for 30 min and stained overnight in carmine alum (0.2% carmine (w/v) and 0.5% aluminum potassium sulfate (w/v)). The mammary glands were then destained (35% HCl, 70% ethanol) for 3-4 hours, dehydrated in increasing concentrations of ethanol and defatted in toluene. Images were captured using a Sony 3CCD color video camera attached to a Leica MZ12 microscope (Leica Microsystems Inc., Buffalo, NY) and Northern Eclipse Software (Empix Imaging Inc. Mississauga, ON, Canada).

In Situ Hybridization. Antisense probes for human IGF-II or IGF-IR were generated by incorporating digoxygenin (DIG)-labeled UTP (Boehringer Mannheim, Laval, PQ, Canada) following the manufacturer's protocol. DIG in situ hybridization was performed essentially as previously described (38,39) with the following modifications. Specifically, tissue was fixed in 4% (w/v) buffered formalin overnight, tissue sections were treated with 20 μg/ml of Proteinase K for 20 min at room temperature and sections were washed 3x in 4x SSC for 15 min each at room temperature.

Western Analysis and Ligand Blotting. Mammary tissue was homogenized in lysis buffer (10 mM Tris pH 7.6, 5 mM EDTA, 50 mM NaCl, 1% triton-X, 30 mM tetra-sodium pyrophosphate, 200 μM sodium orthovanadate, 1mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin) and lysates were collected following centrifugation at 15,000-x g for 20 min at 4°C. Protein concentrations were determined using Bradford assay reagents (Bio-Rad, Hercules, CA). Reduced proteins were separated through 8% polyacrylamide gels using an Xcell II mini cell system (Novex, San Diego, CA). Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 25 V for 90 min and the membranes were blocked in 5% skim milk in tris-buffered saline containing 1% tween (TBST) for 2 hours at room temperature. Primary antibodies for Akt, phosphorylated Akt (Ser473), Erk1/Erk2, and phosphorylated Erk1/Erk2 (Thr202/Tyr204) (New England Biolabs, Beverly, MA) were used at a dilution of 1:1000 in 5% skim milk in TBST while antibodies for p38 MAPK, phosphorylated p38 MAPK (Thr180/Tyr182), ATF-2, phosphorylated ATF-2 (Thr71), Rb, phosphorylated Rb (Ser807/Ser811), and PTEN (New England Biolabs, Beverly, MA) as well as antibody for cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:1000 in 5 % BSA in TBST. The primary antibodies for phosphorylated IRS-1 (Ser612) (Medicorp, Montreal, QU, Canada) and IRS-1 (Upstate Biotechnology, Lake Placid, NY) were used at dilutions of 1:650 and 1:1000 in 5 % skim milk respectively. Membranes were incubated with the appropriate antibody overnight at 4°C. Proteins were detected using an HRP-conjugated anti-rabbit secondary antibody and LumiGLO reagents (New England Biolabs, Beverly, MA) and were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA). Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C. Protein loading was

determined by staining membranes in 1 % amido black in 50 % methanol/10 % glacial acetic acid for 20 min.

Gelatin Zymography. Forty micrograms of mammary protein was separated in an 8% polyacrylamide gel containing 0.1% gelatin. Following electrophoresis the gels were washed briefly in water and then incubated in 2.5% Triton X-100 for 2 hours. The gels were again washed in water and placed in substrate buffer (50mM Tris pH 7.5, 5mM $CaCl_2$, 40mM NaN_3) for 48 hours at 37°C. Gels were stained with Coomassie brilliant blue for 30 min at room temp and destained (50 % methanol, 10 % glacial acetic acid) until the desired intensity was obtained.

Immunohistochemistry. Mammary glands were fixed in 4% (w/v) buffered formalin overnight at room temperature prior to embedding. Paraffin sections were de-waxed in toluene and re-hydrated in decreasing concentrations of alcohol. Sections were digested with Proteinase K (20 μg/ml) at room temperature for 15 min and terminal end-labeling of fragmented DNA in apoptotic cells was performed using an ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY) following the manufacturer's protocol. Bromodeoxyuridine (BrdU) immunohistochemistry was performed as previously described (40).

Elvax-40 Slow Release Pellets. Elvax-40 pellets containing recombinant human IGF-II (rhIGF-II) were generated as previously described (40). Pellets containing 300 ng of rhIGF-II were implanted in the 4th-inguinal mammary gland of wild-type mice at 33 days of age. Control pellets containing only the vehicle (PBS) were implanted in the contralateral mammary gland. The mice were sacrificed 7 days later and the 4th-inguinal mammary glands along with the control or IGF-II pellets were isolated for analysis.

Serum Progesterone and 17-β-Estradiol Levels. Approximately 600-800μl of blood was removed from the mouse at sacrifice. Clotting was permitted for 30 min at room temperature and serum was separated by centrifuging the samples at 6000-x g for 30 min. Serum samples were stored at -70°C until the samples were analyzed for progesterone and 17-β-estradiol were performed by Dr. S. Tokmakejian (University of Western Ontario, Canada) using previously described protocols (41).

Results

Mammary Epithelial Cells Express IGF-II During Development. It is generally accepted that IGF-II expression is repressed in most adult murine tissues except in neural tissue (35,36). Silencing of the IGF-II gene during postnatal development is supported by the fact that IGF-II knockout mice develop postnatally at the same rate as wild type mice. However, a more recent study by Riechert and Wood (34) demonstrated that IGF-II is expressed in adult ductal and alveolar mammary epithelial cells. Our findings using a DIG-labeled antisense IGF-II probe and in situ hybridization, support those of Riechert and Wood in that IGF-II expression could be detected in day 55 developing mammary epithelial cells. IGF-II expression was observed in ductal epithelial cells (arrowhead, Fig. 1A), epithelial cells within TEBs (arrowhead, Fig. 1B) and stromal cells (arrow, Fig. 1A and B) at day 55 of development in wild type mice. IGF-II expression was also detected in epithelial cells from ducts (arrowhead, Fig. 1C) and TEBs (arrowhead, Fig. 1D) in MMTV-IGF-II transgenic mice, albeit at considerably higher levels. Stromal IGF-II expression could also be found in the transgenic mammary tissue (arrow, Fig. 1C). These results demonstrate that IGF-II is normally expressed during pubertal mammary development and that we have been successful in overexpressing IGF-II during this process.

Mammary Development is Delayed in MMTV-IGF-II Transgenic Mice.
Mammary development begins during embryogenesis and continues postnatally until around 10-12 weeks of age. In response to ovarian and pituitary hormones, TEBs form at the tips of the epithelial ducts and ductal lengthening ensues. At day 15 of age a rudimentary ductal tree exists and these epithelial ducts extend into the empty mammary fat pad reaching the lymph node around 35 days of age. Lengthening of the ducts continues until the end of the mammary gland has been reached at around 75 days of age in FVB mice. When the ducts reach the end of the mammary fat pad the TEBs typically disappear (1).

At the whole mount level we observed that ductal length in the fourth inguinal mammary gland was retarded in the IGF-II overexpressors (Fig. 2B,C) compared wild type controls (Fig. 2A) at day 55 of development. When the length of these ducts, extending beyond the lymph node was measured, we found that the epithelial ducts of wild type mice had grown on average 49.1 $\pm$ 3.0 mm beyond the lymph node while epithelial ducts of MI1 and MI12 transgenic lines had extended 35.4 $\pm$ 4.1 mm ($p < 0.05$) and 35.8 $\pm$ 0.7 mm ($p < 0.05$), respectively. In addition to the epithelial ducts being shorter, the number of ducts crossing a line drawn perpendicular to the lymph node and one inch beyond the lymph node, were significantly reduced in both MI1 (12.5 $\pm$ 1.0, $p < 0.05$) and MI12 (11.5 $\pm$ 1.3, $p < 0.05$) mice compared to wild type mice (16.2 $\pm$ 0.9). Further, the amount of the mammary tissue occupied by epithelial cells (data not shown) was consistently reduced in both MMTV-IGF-II transgenic lines.

To investigate whether these differences in epithelial branching arose during a particular period of mammary development or were present at all time points, we examined mammary development at day 35 and day 75 of age. At day 35 no significant differences were observed in the length of the epithelial ducts or the amount of the mammary tissue occupied by the epithelial cells (data not shown). At day 75 of age

there is little difference in epithelial branching between wild type and IGF-II overexpressing mammary glands as the number of ducts in the two tissues is similar. Ductal length cannot be determined at this age as the ducts have reached or nearly reached the end of the fat pad by this time. However, the MMTV-IGF-II transgenic mice have significantly more TEBs remaining in the fourth inguinal mammary gland compared to wild type controls (8.5 ± 1.6 for MI1 vs 2.4 ± 0.9 for Wt, $p < 0.05$ and Fig. 3B vs Fig. 3A). These results suggest that mammary development has not been completed in the IGF-II overexpressors since TEBs generally disappear when ductal elongation has finished. Therefore, mammary morphogenesis during pubertal development, but not prior to, is significantly delayed by IGF-II overexpression.

Recombinant IGF-II Inhibits Mammary Development. An alternative method for elevating IGF-II levels within the mammary tissue was employed to confirm the inhibitory effect of IGF-II on mammary development. Slow-release pellets containing 350 ng of human recombinant IGF-II were implanted into the 4th-inguinal mammary gland of wild type mice near the lymph node at 33 days of age. A control pellet containing only the vehicle was implanted into the contralateral mammary gland and served as an ideal control since both mammary glands are exposed to the same endogenous hormone levels. At 40 days of age, the mammary tissue, along with the pellet, was removed and mammary development was analyzed by whole mount analysis. Mammary development was retarded approximately 20 % in the mammary tissue in which the IGF-II-containing pellet was implanted compared to the control pellet. Thus, local elevation or transgenic overexpression of IGF-II is capable of inhibiting lengthening of mammary epithelial ducts.

Epithelial Proliferation is Reduced in MMTV-IGF-II Transgenic Mice. Extension of the epithelial ducts into the empty mammary fat is dependent on mammary epithelial proliferation and a decrease in proliferation may explain the delay in mammary morphogenesis in the IGF-II overexpressors. BrdU immunohistochemistry was used to determine the percentage of proliferating epithelial cells in wild type (Fig. 3A) and MMTV-IGF-II transgenic (Fig. 3B) mammary tissue. At day 55 of development, mammary epithelial proliferation was significantly reduced in the transgenic mammary tissue (Fig. 3C). The percentage of mammary epithelial cells proliferating was reduced approximately 1.8-fold (6.7 ± 1.0 % for Wt vs 3.8 ± 0.7 % for Tg, $p < 0.05$) when epithelial cells throughout the entire mammary gland were considered. In addition, epithelial proliferation was reduced approximately 1.5-fold in transgenic TEBs compared to wild type TEBs.

Estrogen and Progesterone Levels are Not Significantly Altered in MMTV-IGF-II Transgenic Mice. Mammary ductal morphogenesis is dependent on estrogen, and to a lesser extent, progesterone. To examine whether our phenotype was simply a result of altered levels of 17-β estradiol and/or progesterone, serum levels of these hormones we evaluated. Circulating levels of 17-β estradiol were 432.1 ± 117.4 pmol/L (n=7) in wild type and 404.6 ± 139.2 pmol/L (n=8) in MMTV-IGF-II transgenic mice while the levels of progesterone were 23.4 ± 10.0 (n=7) nmol/L and 35.4 ± 13.1 nmol/L (n=8) in wild type

and MMTV-IGF-II transgenic mice, respectively. These results suggest that the decrease in ductal length was not a result of altered hormone levels.

Gelatinase Activity is Unaffected in MMTV-IGF-II Transgenics. MMP-mediated degradation of the basement membrane surrounding TEBs and epithelial ducts facilitates ductal lengthening and side branching. We have previously demonstrated that shifting the balance in favor of MMP activity through transgenic expression of TIMP-1 antisense RNA resulted in elevated MMP activity and a significant increase in ductal length (40). To examine whether IGF-II overexpression inhibited mammary ductal morphogenesis through a decrease in MMP activity, gelatin zymography on day 55 developing mammary tissue was performed. Both gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in the latent (pro-MMP-9, 105 kDA, pro-MMP-2, 68 kDa) and active (MMP-9, 84 kDa, MMP-2, 60 kDa) forms could be detected in wild type and transgenic developing mammary tissue (data not shown). MMP-2 was the predominant gelatinase at this time with considerably less MMP-9 present. When the MMP profile from wild type mammary tissue was compared to that of two independent MMTV-IGF-II transgenic lines no differences were observed suggesting that IGF-II overexpression did not inhibit mammary ductal lengthening through a decrease in gelatinase activity.

Decreased Epithelial Proliferation in MMTV-IGF-II Transgenic Mice is Associated with a Significant Reduction in Phosphorylated Akt/PKB. In an attempt to identify proteins downstream of IGF-IR that regulated the decreased epithelial proliferation observed in the IGF-II overexpressors, several molecules that have been reported to mediate IGF-IR signal transduction were examined. Using phosphorylation-specific antibodies and western blot analysis we compared the activity of the Erk1/Erk2, p38 MAPK, JNK/SAPK pathways in transgenic and wild type mammary tissue. There were no significant differences in the levels of phosphorylated Erk1/Ekr2 (Fig. 4A), p38 MAPK (Fig. 4B) or ATF-2 (Fig. 4C) in transgenic vs wild type mammary tissue at day 55 suggesting that these proteins are not involved in the IGF-II-mediated inhibition of epithelial proliferation. We were unable to detect phosphorylated JNK/SAPK in the mammary tissue however the phosphorylation status of the transcription factor ATF-2, which is a target of JNK/SAPK, was unaltered in the transgenic mammary tissue.

The only molecule that we found altered in the transgenic mammary tissue was Akt/PKB. We observed a significant reduction in the levels of phosphorylated Akt/PKB in transgenic mammary tissue compared to control tissue (Fig. 4D). Although the activation of Akt/PKB has traditionally been associated with inhibition of apoptosis, more recent studies have shown that phosphorylated Akt/PKB can also promote cell cycle progression through enhancing cyclin D1 expression (42) and protein stability (43). Cyclin D1, complexed with specific cyclin-dependent kinases, can then lead to phosphorylation of Rb, release of the transcription factor E2F and cell cycle progression. To further investigate the consequence of decreased Akt/PKB phosphorylation in the MMTV-IGF-II transgenic mammary tissue the levels of cyclin D1 were examined. We found that transgenic mammary tissue had significantly reduced levels of cyclin D1 (Fig. 4E). Further support for cyclin D1 begin regulated by Akt/PKB phosphorylation stems from the significant positive correlation ($R^2 = 0.43$, $p < 0.05$) between Akt/PKB phosphorylation and cyclin D1 protein levels. In addition, cyclin D1

protein levels were significantly correlated with epithelial proliferation ($R^2 = 0.52$, $p < 0.05$). Together these results suggest that IGF-II overexpression during pubertal mammary development leads to a decrease in Akt/PKB phosphorylation with a subsequent reduction in cyclin D1 protein. Diminished cyclin D1 levels limit cell cycle progression and thus epithelial proliferation and ductal lengthening.

The exact mechanism through which IGF-II overexpression leads to a reduction in Akt/PKB phosphorylation remains unclear. Phosphorylation of Akt/PKB is regulated by factors such as PI-3 kinase that facilitate phosphorylation and molecules such as the phosphatase, PTEN, that inhibit phosphorylation. Since IRS-1 phosphorylation was elevated in the IGF-II overexpressors (Fig. 4F), it is unlikely that a decrease in PI-3 kinase-mediated activation of Akt/PKB can explain the reduction in Akt/PKB phosphorylation. Therefore, we examined the levels of PTEN in transgenic and wild type mammary tissue. We found that the levels of PTEN protein were significantly elevated in the transgenic tissue. Moreover the levels of PTEN protein negatively correlated with Akt/PKB phosphorylation ($R^2 = 0.58$, $p < 0.01$). It is interesting that the wild type sample with the highest level of PTEN protein also had the lowest level of phosphorylated Akt/PKB and the shortest epithelial ducts of the wild type group. Therefore, IGF-II overexpression results in elevated levels of PTEN protein leading to decreased Akt/PKB phosphorylation and cyclin D1 protein levels.

Mammary Morphogenesis During Gestation is Not Affected by IGF-II Overexpression. Gestation is also associated dramatic changes in mammary morphology as the mammary gland prepares for lactation. Ductal epithelial cells proliferate and differentiate into alveolar epithelial cells eventually producing functional lobulo-alveoli. To examine whether IGF-II overexpression affected mammary epithelial proliferation or differentiation during gestation, mammary tissue was analyzed on days 4.5, 8.5, 12.5, 14.5, 16.5, and 18.5 of gestation. In situ hybridization confirmed that IGF-II expression was elevated in the MMTV-IGF-II transgenic mice at all gestational stages examined (data not shown). At the whole mount level there were no apparent differences at any of the gestational stages between wild type and transgenic mammary tissue. To quantify the changes that occur in the mammary tissue during normal gestation and to examine whether IGF-II overexpression altered this process the amount of mammary tissue occupied by epithelial cells, lumens and adipose tissue was determined at the histological level. There was no significant difference in the relative percentage of epithelial cells, lumens or adipose tissue between wild type an IGF-II overexpressing mammary tissue (data not shown). These results indicate that IGF-II overexpression does not affect mammary morphogenesis during gestation.

DISCUSSION

Mammary ductal morphogenesis provides an excellent in vivo system to examine the effects of hormones/growth factors on mammary epithelial proliferation. During embryogenesis a rudimentary ductal tree is generated emanating from the nipple area presumably in response to maternal hormones. Postnatal lengthening of the epithelial ducts remains minimal until the onset of puberty where a surge in circulating levels of ovarian estrogen and progesterone stimulates epithelial proliferation. Ductal lengthening continues until the end of the mammary fat pad is reached. We have used this process of mammary ductal morphogenesis to examine the effects of IGF-II overexpression on mammary epithelial proliferation using MMTV-IGF-II transgenic mice. This system permits the analysis of IGF-II overexpression on mammary epithelial cell proliferation in the context of the appropriate hormone and growth factors milieu that cannot be recapitulated in tissue culture experiments. Although IGF-II is a potent mitogen for mammary epithelial cells in culture our findings indicate that under certain physiological conditions, elevated levels of IGF-II significantly reduced epithelial proliferation resulting in delayed mammary ductal morphogenesis during pubertal development.

IGF-II Overexpression Delays Mammary Morphogenesis Through Inhibition of Epithelial Proliferation. Our results demonstrate for the first time that overexpression of IGF-II inhibited rather than stimulated mammary morphogenesis during pubertal development. This inhibitory effect of IGF-II was observed in MMTV-IGF-II transgenic mice and confirmed through implantation of pellets containing recombinant human IGF-II into developing mammary glands of wild type mice. Inhibition of mammary morphogenesis was characterized by a significant reduction in epithelial ductal length and a decreased number of epithelial ducts at day 55 development as well as a significant increase in the number of TEBs remaining at day 75 of development. The delayed lengthening of epithelial ducts in transgenic mammary tissue was attributed to a significant reduction in epithelial proliferation. We observed a 1.8-fold decrease in the percentage of BrdU positively stained epithelial cells in transgenic mammary tissue compared to wild type controls. Similarly there was a 1.5-fold reduction in the percentage of proliferating epithelial cells in transgenic TEBs. Since epithelial proliferation in TEBs is pivotal for ductal elongation, this reduction in proliferation, at least in part, explains the delay in mammary development in the IGF-II overexpressors.

This finding is intriguing considering that IGFs are potent mitogens for mammary epithelial cells and breast cancer cell lines in vitro (22-27). Further, implantation of IGF-I pellets or systemic IGF-I treatments in estradiol-treated, hypophysectomized, gonadectomized, immature male rats promoted mammary development (44). However, the aforementioned studies represent artificial conditions to study the effect of IGFs on mammary epithelial proliferation. The only other study to examine the effect of IGFs on pubertal female mammary development in vivo was performed by Weber et al. (29). In this study, MMTV-IGF-I transgenic mice were found to have enhanced lobulo-alveolar development in 50 day-old mice that was not observed in wild type controls. Although an increase in lobulo-alveolar development would suggest elevated amounts of epithelial proliferation (Fata and Khokha, unpublished observations), this property was

not assessed in 50 day-old mice. In addition, the effects of IGF-I overexpression on ductal lengthening or TEB formation were not examined. Therefore, overexpression of IGF-I can induce precocious mammary development however its effect on ductal elongation during female pubertal mammary development remains unclear.

Interestingly we have also found that overexpression of the ErbB2 receptor, although associated with increased proliferation and mammary tumorigenesis, significantly inhibited mammary ductal development (Moorehead and Khokha, unpublished data). Thus our findings suggest that factors that promote proliferation may only do so under certain conditions or in specific microenvironments, situations that may be difficult to recapitulate in vitro. Alternatively, inappropriate expression of mitogenic signals may dysregulate the proliferative process resulting in reduced proliferation. Support for the later idea stems from studies in the C/EBPbeta null mice. Loss of the C/EBP gene induced an increase in progesterone receptor expression in the mammary tissue and altered the distribution from a nonuniform to a uniform pattern of expression (45). This alteration in progesterone receptor level and expression pattern inhibited epithelial proliferation and lobulo-alveolar development although the progesterone receptor is normally required for this process. Therefore, inappropriate expression of genes normally involved in stimulating proliferation can under certain physiological conditions, inhibit proliferation.

Mechanism of IGF-II-Mediated Inhibition of Mammary Morphogenesis. In addition to epithelial proliferation, degradation of the extracellular matrix is required for TEB migration, ductal lengthening and the generation of secondary branching (46). Extracellular matrix degradation is regulated by the balance of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Shifting the balance in favor of MMP activity can promote epithelial proliferation and ductal lengthening while increased TIMP activity can inhibit these processes (40). Since IGFs can stimulate MMP production (47-49) we investigated the amount of gelatinolytic activity in transgenic and wild type mammary tissue using zymography. We did not observe any differences in the gelatinolytic activity between transgenic and wild type mice thus suggesting that a decrease in these proteinases did not contribute to the decreased epithelial proliferation and branching in our transgenic mice.

We also investigated whether our phenotype was mediated simply by a change in the circulating levels of estrogen or progesterone since these hormones are critical for normal mammary development and we have detected MMTV-directed IGF-II expression in the ovaries. We found no significant differences in the levels of circulating 17-β estradiol or progesterone between our transgenic and wild type mice suggesting that an alteration in ovarian hormone levels was not responsible for the delayed mammary development in the MMTV-IGF-II transgenic mice. Thus, it appears that overexpression of IGF-II in the mammary tissue was directly responsible for the decreased mammary development rather than acting indirectly through altering either the MMP/TIMP balance or circulating hormone levels.

In an attempt to identify the pathways involved in mediating the growth inhibitory signals from IGF-II, several signal transduction molecules downstream of the IGF-IR were investigated. We examined the phosphorylation status of Erk1/Erk2 and p38

MAPK proteins and found no significant difference in the levels between wild type and transgenic mammary tissue suggesting that these proliferative pathways were not affected by IGF-II overexpression. This finding was not completely surprising as the MAPK kinase proteins mediate proliferative signals from a number of other tyrosine kinase receptors several of which are likely to be activated during mammary development. The JNK/SAPK pathway was also examined but phosphorylated JNK/SAPK could not detected in developing mammary tissue. A potential target of the JNK/SAPK pathway, the ATF-2 transcription factor, was also examined and its phosphorylation status was not significantly different in transgenic mammary tissue indicating that this pathway did not mediate the antiproliferative signals.

The only signaling molecule that we found altered was Akt/PKB. We observed a significant reduction in the levels of phosphorylated Akt/PKB in both of the MMTV-IGF-II transgenic lines examined. Akt/PKB is an antiapoptotic molecule whose properties have been well documented (reviewed in (50-53)). Activation of a number of receptors, including IGF-IR stimulates PI3-kinase activity and subsequent phosphorylation of Akt/PKB. Once phosphorylated Akt/PKB has been proposed to inhibit the proapoptotic molecules Bad and caspase 9. Therefore, the decrease in phosphorylated Akt/PKB in the transgenic mammary tissue would suggest that mammary development was delayed due to a higher rate of epithelial apoptosis. However, we were unable to detect significant levels of apoptosis in the developing transgenic mammary tissue using either in situ end labeling or Hoescht staining. This does not rule out the possibility that the transgenic epithelial cells are more susceptible to apoptotic stimuli and epithelial apoptosis occurred prior to day 55 of development.

More recently, the activation of Akt/PKB has also been shown to stimulate proliferation. D-type cyclins are synthesized during the G_1 phase of the cell cycle and become complexed with cyclin D-dependent kinases. These complexes cause phosphorylation of the retinoblastoma protein and thus stimulate proliferation. Proliferation is halted through phosphorylation of cyclin D1, which targets this protein for degradation. Diehl et al (54) have shown that GSK-3β is one of the molecules that can phosphorylate cyclin D1 and promote its degradation. Since GSK-3β activity can be inhibited by PKB-dependent phosphorylation, PKB promotes cell proliferation by stabilizing cyclin D1 protein levels. In light of the fact that cyclin D1 levels are significantly decreased in our transgenic mammary tissue and this decrease significantly correlates with the levels of phosphorylated Akt/PKB as well as epithelial proliferation implies that IGF-II overexpression inhibits epithelial proliferation by reducing the levels of phosphorylated Akt/PKB and cyclin D1 protein.

It remains unclear exactly how overexpression of IGF-II and presumably increased IGF-IR activation would result in a decreased level of Akt/PKB phosphorylation. It is important to note that Akt/PKB activation is not restricted to signal transduction mediated by the type I IGF receptor and that any receptor that induces PI3-kinase has the potential to influence Akt/PKB phosphorylation. Therefore it is possible that IGF-II overexpression interferes with signal transduction from another receptor. Alternatively, elevated IGF-II expression could modulate another molecule critical for regulating Akt/PKB phosphorylation. One candidate is the phosphatidylinositol 3,4,5-triphosphate, PtdIns (3,4,5)P_3 phosphatase, PTEN (55). Translocation of Akt/PKB and its subsequent phosphorylation is dependent on PI3-kinase-mediated phosphorylation

of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Thus, elevated levels of PTEN would dephosphorylation PtdIns(3,4,5)P_3 and thus prevent Akt/PKB phosphorylation. When the levels of PTEN were examined we observed a significant increase in PTEN levels in the transgenic mammary tissue compared to wild-type tissue. This increased PTEN protein level in the MMTV-IGF-II mice negatively correlated with the level of phosphorylated Akt/PKB and this correlation is significant ($p < 0.05$). It is interesting to note that the one wild type mice with the highest level of PTEN protein had characteristics (Akt/PKB phosphorylation and ductal length) that more closely resembled transgenic mammary tissue than wild type mammary tissue. This observation coupled with the significant negative correlation between PTEN protein levels and ductal length, suggest that PTEN may be an important regulator of mammary morphogenesis. Although the elevated PTEN levels can account for the decrease in phosphorylated Akt/PKB the mechanism through which IGF-II overexpression induces PTEN protein levels is currently under investigation in our laboratory.

Figure Legends

Figure 1. In situ hybridization using a DIG-labeled IGF-II antisense probe in (A,B) an epithelial duct and (C,D) a TEB of (A,C) wild type and (B,D) transgenic mice. Arrowheads and arrows indicate epithelial and stromal IGF-II expression respectively (blue color), scale bar,

Figure 2. Whole mount analysis of the 4^{th}-inguinal mammary gland at day 55 of development in (A) wild type, (B) MI1 transgenic and (C) MI12 transgenic mice, scale bar . Whole mount analysis at day 75 of development in (D) wild type and (E) transgenic mice. Arrowheads indicate club-shaped TEBs, scale bar .

Figure 3. Mammary epithelial proliferation in (A) wild type and (B) transgenic mice as detected by BrdU immunohistochemistry. Arrowheads indicate BrdU positive cells, scale bar . (C) Quantification of the percentage of BrdU positive epithelial cells. Values represent mean ± SEM and values were considered statistically significantly (*) when $p < 0.05$.

Figure 4. Western analysis of (A) Erk1/Erk2, (B) p38 MAPK, (C) ATF-2, (D) Akt/PKB, (E) cyclin D1, (F) IRS-1 and (G) PTEN in wild type and two independent MMTV-IGF-II transgenic lines. Signals were quantified using densitometry and significant differences between wild type and transgenic tissue for (H) phosphorylated Akt/PKB, (I) cyclin D1, and (H) PTEN are shown. Values represent mean ± SEM and Tg-MI1 and Tg-MI12 samples were pooled and presented as Tg. Values were considered statistically significant when $p < 0.05$.

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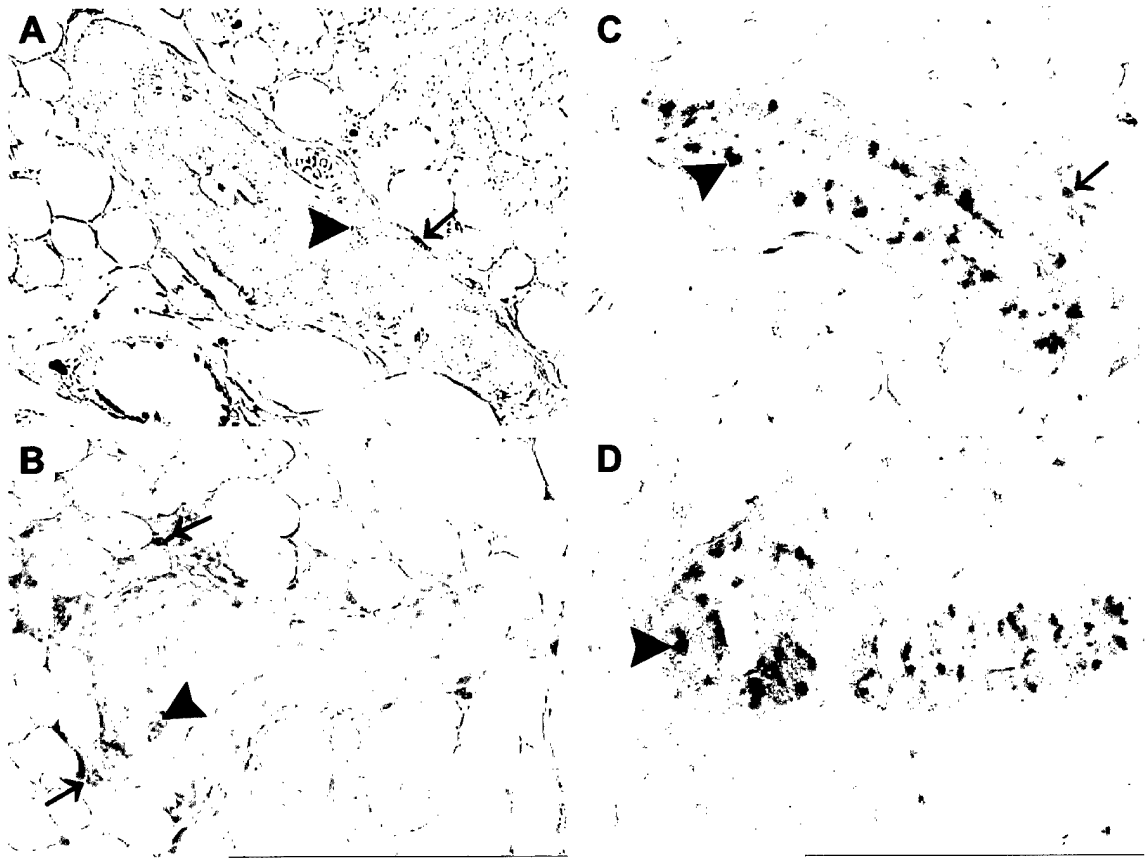
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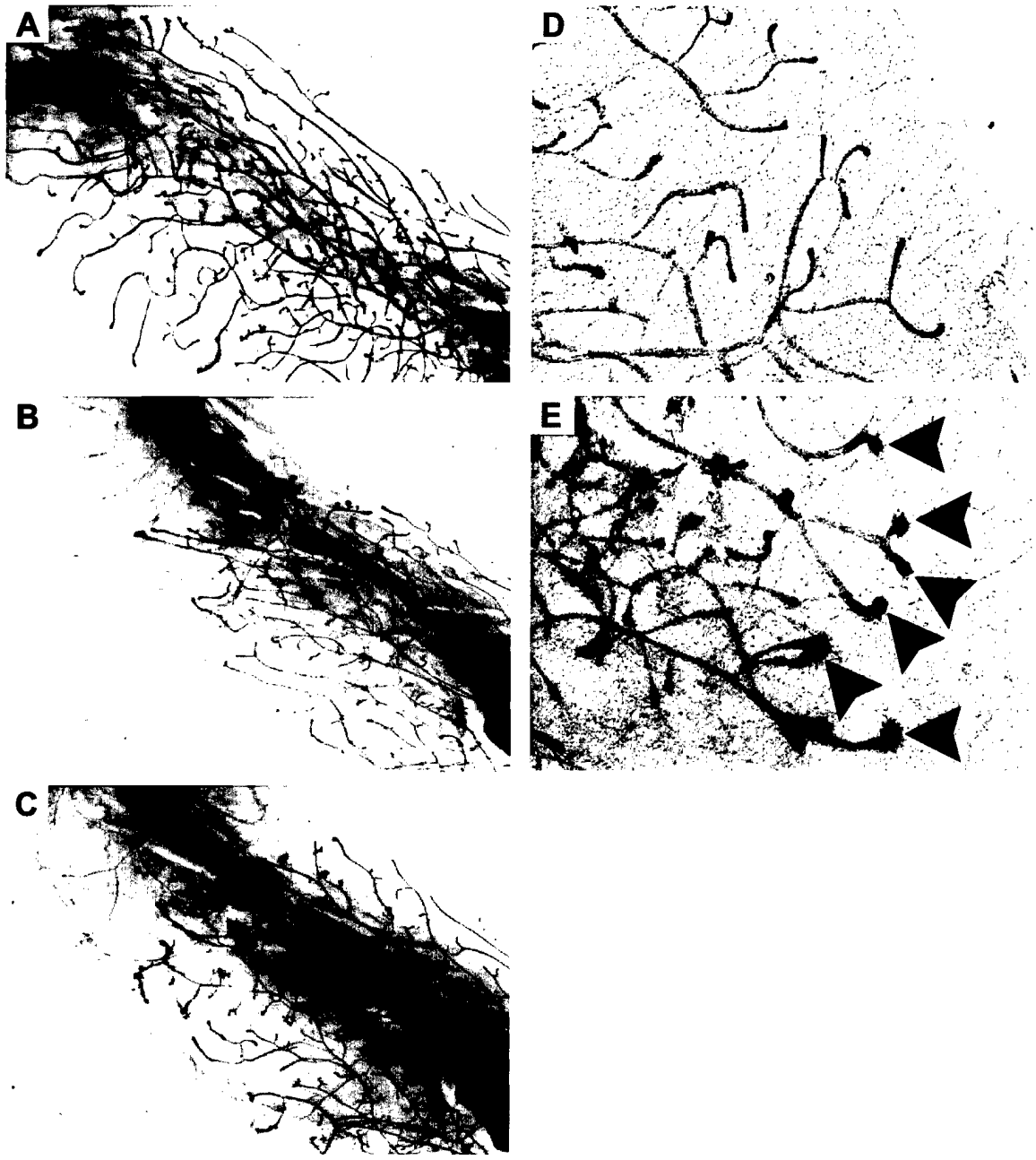

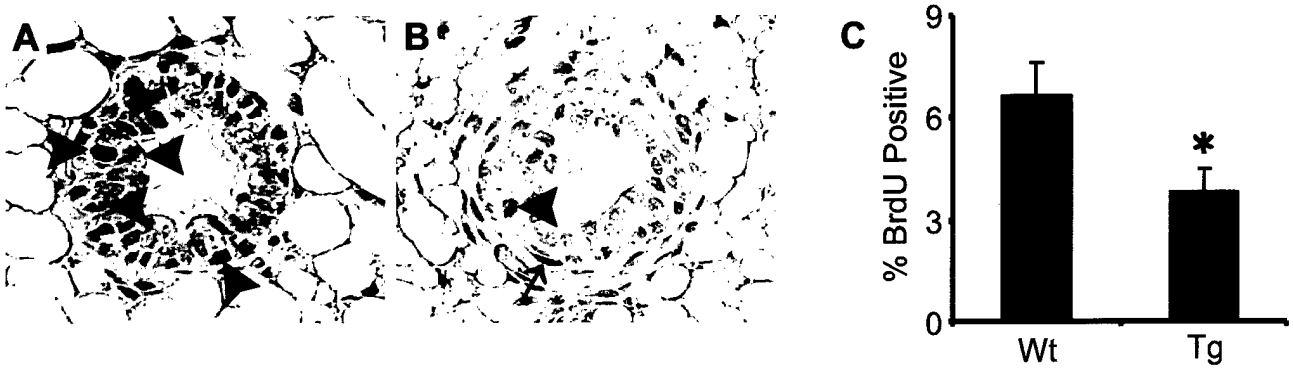
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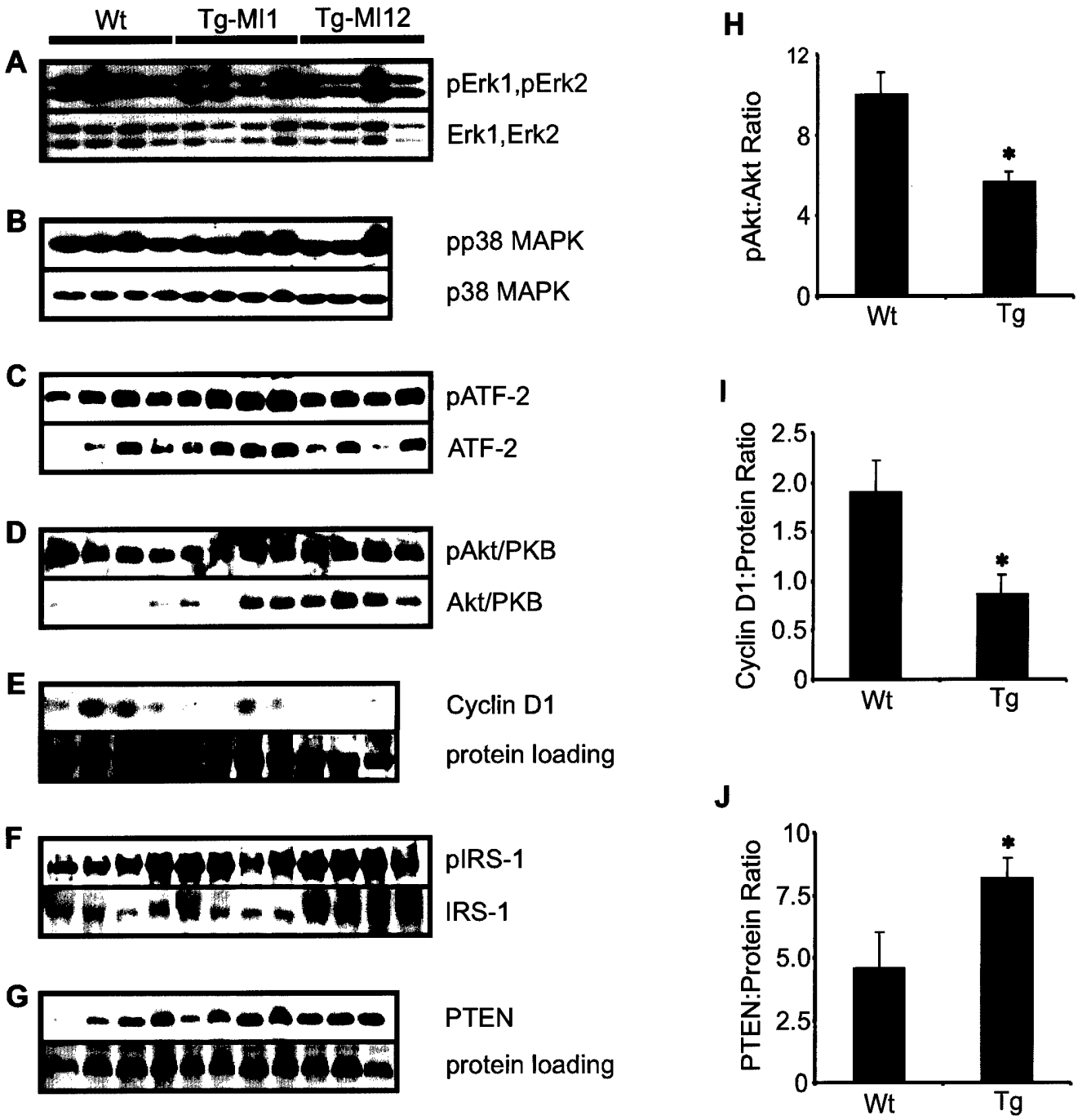
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Inhibition of mammary epithelial apoptosis and sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice

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Running Title: Inhibition of physiological apoptosis by IGF-II

Keywords: IGF-II, transgenic mice, mammary involution, apoptosis, Akt/PKB

ABSTRACT

IGF-II is a growth factor implicated in human cancers and animal tumor models. While the mitogenic properties of IGF-II are well documented, its ability to suppress apoptosis in vivo has never been proven. We generated independent MMTV-IGF-II transgenic mice to examine the control of epithelial apoptosis at the morphological, cellular and molecular levels during the physiological event of postlactation mammary involution. Transgenic IGF-II expression was achieved in mammary epithelium and increased IGF-II bioactivity was confirmed by phosphorylation of the insulin receptor substrate-1, a signaling molecule downstream of the type I IGF receptor. IGF-II overexpression induced a delay in mammary involution, as evident by increased mammary gland to body weight ratios and persistence of both functionally intact lobulo-alveoli and mammary epithelial cellularity. The delayed mammary involution resulted from a significant reduction in mammary epithelial apoptosis, and not from increased epithelial proliferation. Recombinant IGF-II pellets implanted into involuting mammary glands of wild type mice provided further evidence that IGF-II protein inhibited local epithelial apoptosis. At the molecular level, phosphorylated Akt/PKB, but not Erk1 or Erk2, persisted in IGF-II overexpressors and temporally correlated with reduced epithelial apoptosis. Levels of the phosphatase PTEN were unaltered in the transgenic tissue suggesting that the maintenance of Akt/PKB phosphorylation resulted from sustained phosphorylation rather than altered dephosphorylation of PIP-3. Together, this data reveal that IGF-II inhibits apoptosis in vivo and this effect correlates with prolonged phosphorylation of Akt/PKB.

INTRODUCTION

By virtue of their ability to affect cell proliferation as well as cell death, survival factors can play a fundamental role in cancer development. Insulin-like growth factors (IGFs) are considered to be typical examples of survival factors. Components of the IGF axis that include the two ligands (IGF-I and IGF-II), the two receptors (IGF-IR and IGF-IIR), and the IGF binding proteins (IGFBPs) are often dysregulated in several human malignancies including breast cancer (1-3). While the properties of IGF-I that promote tumorigenesis have been extensively studied, the function of IGF-II is not well understood

IGF-I and IGF-II are secreted molecules with distinct characteristics. Although, they elicit their mitogenic and antiapoptotic actions through the same IGF-IR receptor (reviewed in (4)), the two genes differ in vivo in their regulation and function. The levels of IGF-I mRNA increase 10-100 fold in most tissues after birth (5). In contrast, IGF-II mRNA levels are high during embryogenesis but decline during adulthood (6). Circulating levels of growth hormone regulate the hepatic and extrahepatic production of IGF-I, while IGF-II production is not intimately linked to growth hormone levels (7). The significance of IGF-I and IGF-II becomes apparent in animals that lack these genes. Targeted disruption of the IGF-I gene induces death, infertility, and deficiencies in ossification, muscle and lung development, whereas the lack of IGF-II results in viable, fertile, proportionate dwarfs (8-10). Differences between IGF-I and IGF-II also extend to their role in breast cancer. IGF-II mRNA and protein is more frequently detected in primary tumors and human breast cancer cell lines than IGF-I (11). In addition, clinical studies show that stromal cells surrounding normal breast epithelium secrete IGF-I while those surrounding the malignant epithelium secrete IGF-II, suggesting that transformation of breast epithelial cells may be associated with a switch from stromal IGF-I to IGF-II expression (12,13). In rodents, the IGF-II gene is normally imprinted, but is frequently reactivated upon oncogenic transformation in transgenic mice (14,15). Further, the transgenic expression of IGF-II in mouse mammary tissue leads to the development of mammary tumors after long latency (16), indicating a causal link between IGF-II activity and mammary tumorigenesis. It is clear that IGF-II plays a role in modulating breast cancer, however the mechanisms underlying its effects remain to be resolved.

A potential mechanism by which IGF-II can promote mammary tumorigenesis is through inhibiting apoptosis. A large body of in vitro and in vivo evidence demonstrates that IGF-I inhibits apoptosis in several cell types (17-23). On the other hand, the antiapoptotic properties of IGF-II have been shown in vitro, but remain to be proven in vivo. The intracellular molecules responsible for mediating cell survival downstream of the IGF-IR have also not been characterized in vivo. We asked the question whether and how IGF-II overexpression exerts antiapoptotic effects in vivo. To address this, the event of postlactation mammary involution was selected as a model for our studies. Extensive mammary epithelial proliferation and differentiation culminates in the generation of lobulo-alveoli for lactation. However, these structures undergo scheduled regression following the loss of suckling stimuli, accumulation of milk, and decrease in lactogenic hormones. The lobulo-alveolar collapse is a result of massive mammary epithelial apoptosis (24,25). These events can be synchronized by removal of the litter at a specific day of lactation and have been widely studied at the morphological, cellular

and molecular levels (24,26-31). Therefore, postlactation mammary involution offered an appropriate model to uncover the effects of IGF-II on cellular apoptosis and to elucidate the underlying molecular events in a physiological environment.

We generated and characterized three distinct lines of transgenic mice overexpressing IGF-II in mammary epithelial cells by utilizing the MMTV promoter. Increased IGF-II bioactivity was demonstrated through elevated insulin receptor substrate-1 (IRS-1) phosphorylation. We show, genetically and biochemically, that IGF-II reduced mammary epithelial apoptosis and delayed postlactation mammary involution. Molecular analyses of transgenic mammary tissue revealed that the antiapoptotic effects were likely mediated through the sustained phosphorylation of Akt/PKB, an antiapoptotic cytoplasmic protein.

MATERIALS AND METHODS

The MMTV-IGF-II Transgenic Mice. We made an MMTV-IGF-II expression vector by cloning an 833 bp *Pst*I-*Pst*I full-length human IGF-II cDNA (from Dr. K. J. Cullen, Georgetown University) downstream of the MMTV-LTR promoter (from Dr. W. Muller, McMaster University). The MMTV-IGF-II construct also included an untranslated region of the Ha-*ras* gene and a SV40 polyadenylation sequence (Fig. 1A). DNA was prepared for microinjection by digesting the MMTV-IGF-II expression vector with *Sal*I and *Spe*I. The cleaved DNA was electrophoresed through a 1% agarose gel and the transgenic cassette purified with a GeneClean II Kit (Bio/Can Scientific, Mississauga, ON, Canada) using the manufacturer's protocol. The expression construct was microinjected at a concentration of 1 ng/μl into the pronuclei of zygotes from FVB mice and microinjected zygotes were transferred to pseudopregnant FVB females.

Tail biopsies were taken from potential founders and incubated overnight at 55°C in a digestion buffer containing 20 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS and 0.4 mg/ml Proteinase K. Tissue digests were agitated for 5 min using an Eppendorf mixer and 250 μl of 6 M NaCl was added to each sample followed by an additional 5 minutes of mixing. Samples were centrifuged at 20,000 x g for 7 min and DNA was precipitated with isopropanol. Ten micrograms of tail DNA was incubated overnight with *Pst*I at 37°C and the DNA was separated on a 1% agarose gel. Following transfer to Genescreen Plus nylon membrane (NEN Life Science Products, Boston, MA), blots were probed with a [^{32}P]-dCTP-labeled human IGF-II probe. All [^{32}P]-labeled probes used in the present study were generated by random priming using a Prime-It II random primer labeling kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. Membranes were pre-hybridized and hybridized in Church buffer (7% SDS, 1% BSA, 0.5 M NaPi pH 7.2, 0.001 M EDTA) at 65°C. Washes were performed as follows, 0.5% SSC/0.1% SDS for 10 min at room temperature, 0.5% SSC/0.1% SDS for 10 min at 65°C, and 2 times in 0.2% SSC/0.1% SDS for 10 min each at 65°C. Membranes were developed in phosphoimager cassettes (Molecular Dynamics, Sunnyvale, CA) and visualized using a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA)

For routine genotyping, tissue was incubated overnight at 55°C in 50 μl of digestion buffer. The samples were diluted to 400 μl with water and 1 μl was amplified using a forward primer (5'-CCGAGAGGGACGTGTCGA-3') and a reverse primer (5'-GCCTCCCTGAACGCCTCG-3') both located in the IGF-II transgene (primers 2 and 3; Fig. 1A). Forward (5'-CTAGGCCACAGAATTGAAAGATCT-3') and reverse (5'-GTAGGTGGAAATTCTAGCATCATCC-3') primers for the mouse interleukin-2 gene were also included in each reaction and served as a positive control. The IGF-II and interleukin-2 primers produced 187 bp and 324 bp bands, respectively.

RT-PCR and Quantitative RT-PCR. RNA was extracted from mammary tissue using the method of Chomczynski and Sacchi (32). RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco BRL, Burlington, ON, Canada). The resulting cDNAs were amplified using a forward primer (5'-GCCATCCCGTCTCCGCTCGTCACTTATC-3') located in the transcribed segment of the MMTV promoter and a reverse primer (5'-GCCTCCCTGAACGCCTCG-3') located in the IGF-II transgene (primers 1 and 3; Fig. 1A). Forward and reverse primers for interleukin-2 (described above) were used to ensure that each PCR reaction worked.

PCR products were separated on a 1.8% agarose gel and transferred to nylon membrane. Transgene-specific expression was confirmed using a [^{32}P]-dCTP-labeled Ha-*ras* probe (transgene-specific probe, Fig. 1A) and hybridization conditions described above.

The level of transgene expression was determined in mammary glands from 35 day-old female transgenics and their littermate controls using quantitative RT-PCR. RNA was reverse transcribed and amplified using a forward and reverse primer specific for IGF-II transgene (describe above) and a forward (5'-GTTGGATACAGGCCAGACTTTGTTG-3') and reverse primer (5'-GATTCAACTTGCGCTCATCTTAGGC-3') for the house keeping gene hypoxanthine phosphoribosyl transferase (HPRT). It was determined that 23 cycles of PCR fell within the linear range of amplification for both the IGF-II primers and the HPRT primers. Following 23 cycles of PCR, samples were electrophoresed through a 1.8% agarose gel, transferred to nylon membrane and probed with either a [^{32}P]-dCTP-labeled human IGF-II or a [^{32}P]-dCTP-labeled HPRT probe. Membranes were developed and the radioactive signal from IGF-II was quantified relative to that of HPRT using a phosphoimager as described above.

Slot Blot Analysis. Two micrograms of mammary RNA was heated to 68°C for 15 min in a solution containing 50% formamide, 7% formaldehyde and 1X SSC. Two volumes of 20X SSC was added to each sample and the samples were loaded on to nylon membranes (pre-soaked in 20X SSC for 1 hr) using a Minifold II slot-blot system (Schleicher & Schuell, Keene, NH) followed by gentle suction. Each well was rinsed once with 10X SSC followed again by gentle suction. Hybridization of the membrane with the appropriate [^{32}P]-labeled probe and subsequent washes were performed as described above.

In Situ Hybridization. Antisense probes for human IGF-II or Ha-*ras* were generated by incorporating digoxygenin (DIG)-labeled UTP (Boehringer Mannheim, Laval, PQ, Canada) following the manufacturer's protocol. DIG in situ hybridization was performed essentially as previously described (33,34) with the following modifications. Specifically, tissue was fixed in 4% (w/v) buffered formalin overnight, tissue sections were treated with 20 μg/ml of Proteinase K for 20 min at room temperature and sections were washed 3x in 4x SSC for 15 min each at room temperature.

Western Analysis and Ligand Blotting. Mammary tissue was homogenized in lysis buffer (10 mM Tris pH 7.6, 5 mM EDTA, 50 mM NaCl, 1% triton-X, 30 mM tetra-sodium pyrophosphate, 500 μM sodium orthovanadate, 50mM sodium fluoride, 1mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin) and lysates were collected following centrifugation at 15,000 x g for 20 min at 4°C. Protein concentrations were determined using Bradford assay reagents (Bio-Rad, Hercules, CA). Reduced proteins were separated through 8% polyacrylamide gels for Akt, PTEN, and Erk1/Erk2 and 6% gels for IRS-1 using an Xcell II mini cell system (Novex, San Diego, CA). Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 25 V for 90 min and the membranes were blocked in 5% skim milk in tris-buffered saline containing 1% tween (TBST) for 2 hours at room

temperature. Primary antibody for Akt, phosphorylated Akt(Ser473), Erk1/Erk2 and phosphorylated Erk1/Erk2(Thr202/Tyr204) (New England Biolabs, Beverly, MA) were incubated at a dilution of 1:1000 in 5% skim milk in TBST with the membranes overnight at 4°C. The primary phospho-IRS-1 antibody (Ser612) (Medicorp, Montreal, QU, Canada) was used at a concentration of 0.5 μg/ml and the primary IRS-1 antibody (Upstate Biotechnology, Lake Placid, NY) was used at a 1:1000 dilution. The antibody for PTEN (New England Biolabs, Beverly, MA) was used at a dilution of 1:1000 in 5% BSA in TBST. Proteins were detected using an HRP-conjugated anti-rabbit secondary antibody and LumiGLO reagents (New England Biolabs, Beverly, MA) and were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA). Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C. Ligand blotting of wild type and transgenic involuting mammary tissue was performed as we have previously described (14).

Whole Mount Analysis. The 4th-inguinal mammary glands were removed from mice and placed on glass slides. After air-drying for 10 min, the mammary tissue was fixed overnight in Clarke's solution (75% ethanol, 25% glacial acetic acid). Fixed mammary glands were dehydrated in 70% ethanol for 30 min and stained overnight in carmine alum (0.2% carmine (w/v) and 0.5% aluminum potassium sulfate (w/v)). The mammary glands were then destained (35% HCl, 70% ethanol) for 3-4 hours, dehydrated in increasing concentrations of ethanol and defatted in toluene. Images were captured using a Sony 3CCD color video camera attached to a Leica MZ12 microscope (Leica Microsystems Inc., Buffalo, NY) and Northern Eclipse Software (Empix Imaging Inc. Mississauga, ON, Canada).

Immunohistochemistry and Histological Staining. Mammary glands were fixed in 4% (w/v) buffered formalin overnight at room temperature prior to embedding. Paraffin sections were de-waxed in toluene and re-hydrated in decreasing concentrations of alcohol. To detect apoptotic cells in wild type and transgenic involuting mammary tissue, sections were digested with Proteinase K (20 μg/ml) at room temperature for 15 min and terminal end-labeling of fragmented DNA in apoptotic cells was performed using an ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY) following the manufacturer's protocol. Detection of apoptotic cells in the pellet experiments and bromodeoxyuridine (BrdU) immunohistochemistry were performed as previously described (35,36).

Elvax-40 Slow Release Pellets. Elvax-40 pellets containing recombinant human IGF-II (rIGF-II) (Calbiochem, San Diego, CA) were generated as previously described (36). Pellets containing 500 ng of rIGF-II were implanted in the 4th-inguinal mammary gland of wild-type mice at day 2 of involution. Control pellets containing only the vehicle (PBS) were implanted in the contralateral mammary gland. The mice were sacrificed three days later and the 4th-inguinal mammary glands along with the control or IGF-II pellets were isolated for analysis.

Statistics. All values are presented as mean ± SEM. Statistical significance was determined using the Student's t test and values were considered significant when $p < 0.05$.

RESULTS

Transgenic IGF-II Expression in Mammary Epithelium of MMTV-IGF-II Mice. To derive transgenic mice expressing elevated levels of IGF-II, an expression construct containing the full-length human IGF-II (hIGF-II) cDNA under the control of the MMTV-LTR (Fig. 1A) was microinjected into one-cell zygotes. Founder animals were identified by probing Southern blots of *Pst*I cleaved tail DNA with a hIGF-II DNA probe which detected an 833 bp transgene-specific (t-IGF-II) fragment, as well as a 1391 bp endogenous IGF-II (e-IGF-II) fragment (Fig. 1B). Three male founders were identified (MI1, MI12 and MI16) and an independent transgenic line established from each founder mouse.

Next we screened for IGF-II expression in the mammary tissue. Mammary mRNA from 35 day-old wild type and transgenic mice was analyzed by RT-PCR using both transgene-specific and control primer sets. A forward primer located in the transcribed portion of the MMTV promoter and reverse primer in the IGF-II transgene (primers 1 and 3, Fig. 1A) produced 600-800 bp fragments (t) that were evident only in the mammary tissue of transgenic mice (Fig. 1C). The 296 bp fragment generated from the interleukin-2 control (c) primers was visible in every lane and served as a positive control for individual PCR reactions. To further verify that the 600-800 bp fragments were indeed the result of transgene expression, PCR fragments were transferred to nylon membrane and probed for the presence of the Ha-*ras* sequence that is present in the 5' untranslated region of the expression construct. As shown in Fig. 1C, Ha-*ras* expression (t*) was detected only in transgenic mammary tissue.

To assess whether transgenic IGF-II expression was restricted to the mammary tissue, RT-PCR analysis was performed on a number of organs. We found that salivary gland, spleen, and uterus also expressed t-IGF-II in all three independent transgenic lines while kidney, ovary, liver and pancreas, expressed t-IGF-II in some of the lines (data not shown). These organ-specific expression profiles were consistent with the literature describing MMTV-driven transgenes (37,38). To compare the level of t-IGF-II expression among the three transgenic lines, quantitative RT-PCR was performed utilizing primer sets for the IGF-II transgene (primers 2 and 3; Fig. 1A) and endogenous HPRT. The latter served as an internal control for PCR amplification. These analyses indicated that both MI1 and MI12 mice had considerably higher transgene expression than MI16 mice (data not shown). Thus, we had established three independent MMTV-IGF-II transgenic lines with different levels of ectopic t-IGF-II expression in the mammary gland.

To identify the cell types responsible for IGF-II production during involution, in situ hybridization was performed on mammary tissue isolated from day 3 of involution (d3i) using strand-specific IGF-II or Ha-*ras* riboprobes. IGF-II-specific signal was detected in epithelial cells of wild type tissue, albeit at very low levels (Fig. 1D), which is consistent with the findings of Richert and Wood (39). The transgenic mammary tissue showed IGF-II-specific signal, which localized to epithelial cells (Fig. 1E). This signal was much more intense than the e-IGF-II and was present in a majority of epithelial cells. In situ hybridization with a Ha-*ras* riboprobe was used to confirm that the transgene expression was confined to the epithelial cells of transgenic animals (Fig. 1F,G). Given that stage- and site-specific expression of IGF-II occurred appropriately for our studies, these mice were subjected to further molecular analyses.

Increased IGF-II Bioactivity in MMTV-IGF-II Mammary Tissue. To gauge the amount of transgenic IGF-II in relation to endogenous IGF-II, the levels of e-IGF-II and t-IGF-II mRNA were analyzed in wild type and transgenic mice during involution by northern blot and slot blot analyses. Very low levels of e-IGF-II mRNA were evident in mammary tissue from wild type mice and these levels were maximal on days 10 of lactation and 1 of involution (Fig. 2A). On the other hand, transgenic IGF-II mRNA was observed throughout involution, and its expression was 50-100 fold that of the e-IGF-II mRNA levels (Fig. 2A). Thus the level of t-IGF-II was far greater than the e-IGF-II.

The IGFBPs play an important function in the IGF axis. They serve to transport IGFs and modulate their half-life, as well as regulate IGF:IGF receptor interactions (reviewed in (40,41)). Thus, the levels of IGFBPs in a given tissue can influence IGF-II bioavailability. A 50-fold increase in IGFBP-5 and smaller increases in IGFBP-2 and IGFBP-4 have been reported in the rat mammary tissue on day 2 of involution (42). We evaluated whether the levels of IGFBPs were altered in the IGF-II overexpressing mammary tissue, since a concomitant change in these binding proteins would influence the availability of t-IGF-II. Ligand blot analysis revealed that an IGFBP of ~32 kDa (possibly IGFBP-5) peaked at d2i and remained detectable until d4i in both genotypes (Fig. 2B). Since the levels were comparable in transgenic and wild type mice, we concluded that the increased IGF-II production in the transgenic mammary tissue was not confounded by altered IGFBP levels in the tissue. Overall, an increased amount of IGF-II was available to interact with its receptors in the MMTV-IGF-II mammary tissue.

IGF-II induces signal transduction primarily through the IGF-IR (43), although it has also been reported to stimulate insulin receptor signaling (44,45). The IRS-1 protein binds to activated IGF-IR, as well as to activated insulin receptor, becomes phosphorylated and propagates signal transduction from both of these receptors. We used immunoblotting with specific antibodies to monitor the levels of phosphorylated IRS-1 (p-IRS-1) and total IRS-1 protein. The ratio of p-IRS-1 to IRS-1 was taken as an indicator of t-IGF-II bioactivity in the transgenic tissue. The levels of p-IRS-1 were considerably higher in IGF-II overexpressing mammary tissue obtained at 10L, compared to that from controls (Fig. 2C). Specifically, the ratio of p-IRS-1 to total IRS-1 in the transgenic tissue was 2.5-fold that of the wild type tissue (Fig. 2D). An increase in the level of p-IRS-1 was also evident in 75-day-old virgin transgenic mammary tissue (data not shown). Together, these observations show that mammary overexpression of IGF-II resulted in increased IGF-II bioactivity in the MMTV-IGF-II mice.

Delayed Mammary Involution in MMTV-IGF-II Transgenic Mice. Since extensive epithelial apoptosis is responsible for postlactation mammary involution, we selected this process as a model to delineate the impact of t-IGF-II expression on apoptosis in vivo. The following measures were implemented to ensure consistency in this system. We minimized the mouse-to-mouse variation that can arise from the suckling response by maintaining the litter size at 5 pups per female, and synchronized the onset of mammary involution by removing litters on day 10L. In addition, all mice were analyzed following their first pregnancy.

As an initial measure of the gross mammary alterations that ensue during involution, the weight of 4th-inguinal mammary fat pads relative to the body weight of the

mouse, were monitored. An initial increase in this value was expected after pup removal due to milk accumulation, and was observed in both wild type and transgenic mice at d1i. As involution proceeds, this ratio declines in a characteristic manner leveling out around d4i, a time period of maximal epithelial apoptosis. We found that the mammary gland-to-body weight ratio was consistently higher from d1i to d4i in the MMTV-IGF-II mice compared to wild type controls and these differences were significant at several of the time points (Fig. 3A).

To ascertain whether elevated expression of IGF-II resulted in morphological alterations in the epithelial ductal structures, whole mount analyses were conducted on each day beginning at 10L until d8i. At day 10L, the mammary gland is packed with large, milk-filled lobulo-alveoli. Progressive, scheduled involution then leads to lobulo-alveolar regression that is followed by the clearing of mammary epithelial cellularity and the reconstitution of adipose tissue. We found that the process of mammary involution was delayed in IGF-II overexpressing mice. Specifically, the bud-like, lobulo-alveolar structures persisted for a longer period before regression in MI1 transgenics and these differences were greatest at d4i (Fig. 3B vs 3C). It was clearly evident that many lobulo-alveoli were still dilated and contained a secretory substance in the transgenic mice (arrowhead, Fig. 3B), whereas these structures had regressed in wild type mice (Fig. 3C). Further, an increase in β-casein mRNA expression relative to 18S rRNA expression was observed in transgenic mammary tissue at 10L and d1i (data not shown). This suggested that the lobulo-alveoli in the transgenic mice also differed functionally from those in control mice.

Next we performed histological analyses to assess the differences at the cellular level between transgenic and wild type tissue. The mammary tissue has distinct compartments; connective tissue composed of adipocytes and extracellular matrix and parenchymal tissue composed of epithelial and myoepithelial cells. At 10L, the mammary gland is composed entirely of a parenchymal compartment with functional lobulo-alveoli and this compartment is removed via apoptosis during mammary involution. Typically, lobulo-alveoli begin to collapse around d3i and cords of mammary epithelial cells predominate by d5i. We performed two measurements: first, we determined the percentage of mammary tissue occupied by the parenchymal compartment and found this value to be increased in involuting transgenic mammary tissue on days spanning d3i-d6i. Significant differences between MI1 transgenic and wild type mice were observed on d5i and d6i ($23.6\% \pm 1.6$ vs $11.3\% \pm 2.1$, $p < 0.005$ on d5i; and $14.9\% \pm 1.5$ vs $6.9\% \pm 1.2$, $p < 0.01$ on d6i). Second, we compared the number of structurally intact lobulo-alveoli remaining on specific days of involution. Lobulo-alveolar regression normally occurs earlier in the post-nodal area (from the end of the mammary fat pad to lymph node) than in the pre-nodal area (from lymph node to teat). To maintain consistency we quantified the numbers of lobulo-alveoli in the post-nodal area. Lobulo-alveolar collapse was clearly delayed in MI1 transgenic mammary glands at d4i (Fig. 3D vs 3E) and a higher number of lobulo-alveoli were present from d3i to d6i in the mammary glands of transgenic mice than of controls (Fig. 3H). This phenotype was not a result of increased epithelial proliferation as the percentage of BrdU positive epithelial cells was not significantly different in the transgenic mammary tissue (data not shown). To rule out the possibility that these phenotypes arose from events other than increased IGF-II expression, such as the site of transgene integration,

independent transgenic lines (MI12 and MI16) as well as the MI1 double hemizygous (carrying two IGF-II alleles) mice were subjected to similar analyses on day 5 of involution. An elevation in the mammary cellularity, as indicated by the parenchyma compartment was observed in MI12 (23.6%) and MI16 (26.6%) mice and this value was further increased in MI1 double hemizygous transgenics (37.4%). In addition, multiple intact lobulo-alveoli persisted in MI16 (Fig. 3F) and MI1 double hemizygotes (Fig. 3G) at d5i, a time when the majority of these structures have regressed in wild type mice. This demonstrated that the phenomenon of delayed mammary involution occurred consistently in independent MMTV-IGF-II lines.

Decreased Mammary Epithelial Apoptosis in MMTV-IGF-II Mice. To determine the basis of increased mammary cellularity in the transgenic tissue, we elucidated the apoptotic index of mammary epithelial cells throughout involution by immunohistochemistry (Fig 4A,B). It is known that programmed cell death is responsible for lobulo-alveolar collapse and removal of epithelial cells. We observed two classes of lobulo-alveolar structures, those that had collapsed and consisted primarily of apoptotic cells, and those appearing essentially intact and consisted primarily of viable epithelial cells. Separate measurements were performed for all apoptotic epithelial cells within the mammary gland and for apoptotic epithelial cells within intact lobulo-alveoli. Kinetics of apoptosis during scheduled involution in wild type mice showed that apoptotic cells were first detected at d1i and peaked at d4i, which was consistent with published reports (27,30,46). The percentage of total mammary epithelial apoptosis in MMTV-IGF-II mice lagged behind that of the controls (data not shown). When intact lobulo-alveoli were considered, apoptotic cells could not be detected until d2i (Fig. 4C) in the IGF-II overexpressors whereas apoptotic epithelial cells were observed at d1i in wild type mice. Further, the numbers of apoptotic cells were approximately half of those observed in the control tissue, from d3i to d5i ($p < 0.05$, Fig. 4C).

Decreased Epithelial Apoptosis Correlates with Sustained Phosphorylation of Akt/PKB. We further explored the molecular basis of the antiapoptotic effect of IGF-II. A number of in vitro studies have shown that IGF-I and IGF-II inhibit apoptosis through the activation of Akt/PKB (47-50). We investigated whether reduced mammary epithelial apoptosis in transgenic tissue was linked to activation of Akt/PKB. Western blots for Akt and p-Akt spanning 10L to 4di from two independent sets of animals are presented in Figure 5A. Phosphorylated Akt/PKB was indeed detected at 10L in both wild type and transgenic mice and its levels increased approximately 5-fold at d1i (Fig. 5B). In wild type mice, the levels of p-Akt/PKB diminished rapidly by d2i and were undetectable thereafter. It was striking that the levels of phosphorylated Akt/PKB remained elevated until d3i and were observed as late as d4i in the MMTV-IGF-II mice. Sequential probing of the western blots provided a measure of total Akt/PKB in the tissue.

Akt/PKB phosphorylation is regulated by the phosphorylation status of the lipid phosphatidylinositol (3,4,5)-triphosphate (PIP-3). Since the phosphatase PTEN can dephosphorylate PIP-3, levels of PTEN can limit the duration of Akt/PKB phosphorylation (51-53). There were no differences in the levels of PTEN protein in the

wild type versus the transgenic mammary tissue (Fig. 5C). Interestingly, it appeared that the levels of PTEN protein began to increase at d3i, the time the Akt/PKB phosphorylation was lost.

Although the downstream targets of Akt/PKB are still poorly understood it has been demonstrated that Akt/PKB can phosphorylate Bad in tissue culture systems (reviewed in (54-56)). We attempted to assess the phosphorylation status of Bad in our mammary tissue. The phosphorylation-specific antibody (Ser112) was unable to detect phosphorylated Bad during the events of physiological apoptosis (data not shown).

Activation of Erk1 and Erk2 has also been implicated in inhibiting apoptosis, possibly through phosphorylation of Bad (57,58). Since IGF-II-mediated signaling through the IGF-IR can stimulate the Erk pathway (4) we examined the levels of phosphorylated Erk1 and Erk2 to seek the involvement of this pathway in the antiapoptotic response of IGF-II. The levels of phosphorylated Erk1 and Erk 2 were lowest at 10L and increased as involution proceeded. There were no differences in Erk1 or Erk2 in the wild type and transgenic involuting mammary tissue. If these proteins had a function in the physiological antiapoptotic response of IGF-II, higher levels of phosphorylated Erk1/Erk2 at 10L to d2i would be expected. Together, this data revealed Akt/PKB as the likely molecule in the antiapoptotic signaling of IGF-II in vivo.

Normal Postlactation Epithelial Apoptosis is inhibited by IGF-II Slow-Release Pellets. Low levels of IGF-II exist during murine mammary morphogenesis (39), and we had observed low IGF-II expression during the period of postlactation involution (Fig. 2A). To investigate whether elevating IGF-II during normal mammary involution would directly inhibit mammary epithelial apoptosis, a biochemical approach was used with topical application of recombinant IGF-II. Elvax-40 slow-release pellets containing 500 ng of rIGF-II were implanted in the 4^{th}-inguinal mammary gland of wild type mice on d2i, in the post-nodal area. An inert control pellet that lacked IGF-II was implanted in the contralateral tissue of each mouse. Due to the identical hormonal milieu of the two glands, the contralateral tissue serves as an ideal control. Epithelial apoptosis surrounding either a pellet containing rIGF-II or a control pellet, detected immunohistochemically, is shown in Figure 6A and 6B respectively. A significant decrease in the apoptotic index of epithelial cells in the immediate vicinity of the implanted rIGF-II pellets was found in tissue removed at d5i (Fig. 6C). This provided an independent confirmation that an elevation of IGF-II protein directly inhibited the normal mammary epithelial apoptosis.

DISCUSSION

We have generated independent MMTV-IGF-II transgenic mice and examined epithelial apoptosis at the morphological, cellular and molecular levels during the physiological event of postlactation mammary involution. Here we provide the first in vivo demonstration of the anti-death role of IGF-II during physiological apoptosis. The antiapoptotic effect of IGF-II was initially evident in the involuting mammary glands as a delayed onset of mammary weight loss, slower lobulo-alveolar involution of mammary tissue at the morphological level, and higher epithelial cellularity at the tissue level, when compared to controls. This resulted from a delayed onset of epithelial apoptosis rather than altered proliferation. The antiapoptotic effect of IGF-II was confirmed biochemically. Elevating IGF-II protein levels through implantation of a slow-release pellet directly led to a local reduction of epithelial apoptosis within the involuting tissue. Downstream of the IGF-IR, sustained phosphorylation of Akt/PKB throughout the period of delayed epithelial apoptosis confirmed that this negative regulator of apoptosis contributed to the antiapoptotic effects of IGF-II in vivo. This study provides further evidence that the contribution of IGF-II to breast tumorigenesis may extend beyond it known proliferative capacity.

Role of the IGF Axis in Mammary Involution. The survival of mammary alveolar epithelial cells during lactation is dependent on a number of factors including prolactin and growth hormone. It was originally proposed that growth hormone suppressed alveolar epithelial apoptosis through elevating the levels of IGF-I and/or IGF-II (59). However, subsequent studies in which rats were subcutaneously administered IGF-I, IGF-II or IGFs complexed with IGFBP-3, failed to mimic the survival effects of growth hormone during involution (60,61). Such an inability of the IGFs to inhibit apoptosis has been attributed to changes in the levels of IGFBPs that concomitantly occur during involution (42,62). It is thought that IGFs present in the milk act as survival factors, but the elevation of IGFBPs during involution effectively sequester the IGFs. This prevents the interaction of IGFs with the IGF-IR, essentially removing the IGF survival signal from mammary epithelial cells (42,62). In support of this concept are the findings that when sufficient levels of IGF-I are attained through transgenic overexpression, IGF-I suppresses apoptosis and promotes epithelial cell survival during mammary involution (17,63).

The involvement of IGF-II in mammary involution has not been studied. Until a recent publication by Richert and Wood (39) it was generally believed that IGF-II was not expressed in adult murine mammary tissue (8,64). Our in situ hybridization with a riboprobe specific for IGF-II extended the findings of Richert and Wood (39) in that IGF-II was also expressed during postlactation involution within mammary epithelial cells, albeit at low levels. Further, we found that the levels of endogenous IGF-II increased from 10L to d1i and declined thereafter, suggesting that it may play a role in mammary involution. The decrease in endogenous IGF-II expression after d1i, coupled with a dramatic increase in IGFBP levels at d2i, likely removes the IGF-II-mediated survival signals. In our genetic approach, much higher levels of IGF-II were achieved throughout involution in the transgenic tissue. This IGF-II expression, in the absence of concurrent IGFBP elevation, maintained IGF-II-mediated survival signaling and delayed mammary epithelial apoptosis and lobulo-alveolar collapse. The same effect was

obtained when a biochemical approach was used. Implantation of IGF-II pellets in the mammary tissue of wild type mice inhibited local mammary epithelial apoptosis. These observations highlight the potential for IGF-II, like IGF-I, to regulate natural mammary involution.

The Antiapoptotic Property of IGF-II may Underlie its Tumorigenic Potential. Based on previous studies in transgenic systems, a causal relationship has emerged between IGF-II expression and cancer formation in several tissues (reviewed in (11)). For example, the transgenic expression of IGF-II has been associated with lymphomas and hepatocellular carcinomas ((65) and reviewed in (66)), and its expression from a mammary-specific sheep β-lactoglobulin promoter leads to mammary tumorigenesis (16). Further, the normally silent IGF-II gene often becomes reactivated during viral oncogene-driven tumorigenesis, such as that observed in pancreas (15) and liver (14). While IGF-II expression is temporally coordinated with the onset of proliferation in some of these models (14,15,67), it is conceivable that its antiapoptotic property also plays a crucial role in the genesis of cancer. This may be especially applicable to the mammary gland since this tissue experiences repeated and extensive epithelial turnover throughout female life, during each round of the estrous cycle ((68), Fata and Khokha, unpublished data) and postlactation involution (24,26-31).

Our investigations used both genetic and biochemical approaches to conclusively show that IGF-II suppressed mammary epithelial apoptosis in vivo during postlactation mammary involution. Based on these observations we propose that the low incidence of mammary tumorigenesis seen in IGF-II transgenics such as the β-lactoglobulin-IGF-II mice (16), may be initiated by incomplete epithelial apoptosis, a hypothesis that was not examined by this group. It has been shown that reduced mammary epithelial apoptosis due to Bcl-2 overexpression, although insufficient to confer tumorigenesis, enhances the development of MMTV*myc*-induced mammary tumors (69). These findings of reduced apoptosis are relevant since decreased mammary epithelial apoptosis is associated with an increased risk of fibrocystic changes and the development of carcinoma in premenopausal women (70). We are currently breeding our MMTV-IGF-II transgenic mice with mice that overexpress the *neu* oncogene to examine whether IGF-II-mediated inhibition of epithelial apoptosis augments *neu*-induced mammary tumorigenesis.

In Vivo Molecular Targets of IGF-II Survival Signaling. We extended our finding of IGF-II-mediated inhibition of mammary epithelial apoptosis to the molecular level by examining downstream antiapoptotic signaling molecules. In vitro, the IGFs have been shown to inhibit apoptosis through the activation of Akt/PKB (47-49). Our analyses of the total and phosphorylated levels of Akt/PKB revealed that p-Akt/PKB was detectable in MMTV-IGF-II mammary tissue until d4i, while it disappeared after d2i in wild type mice. The temporal decline of p-Akt/PKB coincided with the peak of epithelial apoptosis during involution in both transgenic and wild type mice. Specifically, minimal levels of apoptosis were observed at times of high levels of p-Akt/PKB, and high levels of mammary epithelial apoptosis were seen immediately after the levels of p-Akt/PKB had substantially declined. Thus, the dynamics of Akt/PKB phosphorylation were consistent with its role as a negative regulator of apoptosis.

The dephosphorylation of Akt/PKB may, in part, relate to the changing levels of IGFBPs. Consistent with our finding that IGFBP levels peaked at d2i in the mouse, a dramatic increase in IGFBPs has been reported at day 2 of involution in rats (42). Day 2i is also the time when we observed a rapid decline in the levels of p-Akt/PKB in wild type mice. An increase in IGFBP levels at d2i likely reduced IGF bioavailability, which in turn, limited the downstream phosphorylation of Akt/PKB. Based on these findings we propose that t-IGF-II bioactivity and Akt/PKB phosphorylation was sustained in the MMTV-IGF-II transgenic tissue until d4i due to the high IGF-II:IGFBP ratio. The fact that IGFBP levels were comparable to those in wild type mice while IGF-II expression was increased in the MMTV-IGF-II mice likely resulted in an abundance of free IGF-II available to stimulate IGF-IR and activate Akt/PKB.

Another potential regulator of Akt/PKB phosphorylation is the phosphatase PTEN. This molecule dephosphorylates PIP-3, which is then unable to promote Akt/PKB activation (51-53). We did not observe a differential regulation in the levels of PTEN protein among the wild type and transgenic mice suggesting that the maintenance of Akt/PKB phosphorylation was primarily driven by the increased availability of IGF-II rather than a reduction in the levels of PTEN. The eventual loss of Akt/PKB phosphorylation in transgenic mice may relate to the dependence of mammary epithelial cell survival on other parameters such as the contact with the extracellular matrix molecules, as discussed in the next section.

Exactly how Akt/PKB inhibits apoptosis remains unresolved. It has been proposed that Akt/PKB phosphorylates the proapoptotic molecule Bad and prevents its interaction with Bcl-2 thus suppressing cytochrome *c* release from mitochondria and subsequent apoptosis (56). We were unable to detect phosphorylated Bad in our involuting mammary tissue. This result was not completely unexpected as Bad is expressed at very low levels (56). In humans, another proposed downstream antiapoptotic target of Akt/PKB is caspase 9. Akt/PKB can phosphorylate human caspase 9 on Ser196 and inhibit its protease activity (71), however, this phosphorylation site is absent in mice (56) and thus the relevance of caspase 9 in mediating the antiapoptotic effects of Akt/PKB in murine tissue is unclear.

IGF-II also has the potential to inhibit apoptosis through the activation of Erk1 and Erk2 (57,58). Since there was no difference in the levels of phosphorylated Erk1 or Erk2 in our transgenic mammary tissue it appears that this pathway does not significantly contribute to the antiapoptotic effects of IGF-II during involution. To our knowledge, this is the first study to demonstrate that IGF-II inhibits epithelial apoptosis in vivo, an effect likely mediated by the sustained activation of Akt/PKB.

The Antiapoptotic Effects of IGF-II are Influenced by Structural Factors. Both soluble factors and contact with the basement membrane are critical for epithelial cell survival (24,26,27,72,73). When mammary epithelial cells are cultured on basement membrane, apoptosis induced by the withdrawal of lactogenic hormones can be inhibited by the addition of IGF-I or IGF-II (74). Also, IGF-mediated phosphorylation of IRS-1 and its association with PI-3 kinase are enhanced in cells cultured on basement membrane suggesting that antiapoptotic signaling from the IGF-IR is more efficient when mammary epithelial cells are in contact with the basement membrane (74). Further, the IGFs are incapable of preventing apoptosis in the presence of an antibody

that blocks the binding of mammary epithelial cells to laminin (74). Overall these studies indicate that soluble factors, such as the IGFs, act in concert with structural signals, such as the extracellular matrix, in determining epithelial apoptosis. This also likely explains why transgenic IGF-II expression only delayed mammary gland involution and did not completely inhibit this process. The role of basement membrane degradation during involution and the regulation of the cleavage of IGFBPs by the matrix metalloproteinases, and the inhibition of this process by tissue inhibitors of metalloproteinases are currently under investigation in our lab.

Phenotypes in IGF-II Overexpressing Transgenic Mice. Human IGF-II has been used to generate a number of transgenic strains that develop organ abnormalities as well as lymphomas, hepatocellular carcinomas and lung adenocarcinomas (65,75-83). With respect to IGF-II overexpression in the mammary tissue, Bates et al (84) have used the sheep β-lactoglobulin promoter and demonstrated mammary tumor formation albeit at a relatively low tumor incidence. This promoter induces transgene expression primarily during lactation (85-87). In contrast, our use of the MMTV promoter resulted in transgene expression at all stages of mammary physiology: ductal development, lactation and involution. Our oldest mice are ~18 months and have yet to show overt mammary tumors. However, we have observed focal areas of epithelial hyperplasia in mammary glands of multiparous transgenic mice as well as sporadic tumors in other organs where MMTV-directed IGF-II is expressed (Moorehead and Khokha, unpublished observations). At present, it remains unresolved why IGF-II overexpression driven by the β-lactoglobulin promoter but not the MMTV promoter induces mammary tumors. It is difficult to compare the β-lactoglobulin-IGF-II and MMTV-IGF-II mice since the basal level of transgene expression in the mammary tissue prior to tumor formation and the number of pregnancies required has not been reported for the β-lactoglobulin mice (16).

In summary, this study represents the first report on the ability of IGF-II to inhibit physiological apoptosis. We also show that this antiapoptotic effect correlates with sustained phosphorylation of Akt/PKB and not Erk1 or Erk2. Based on the observation that PTEN protein levels are unaltered, we propose that the sustained phosphorylation of Akt/PKB in the MMTV-IGF-II transgenics was propagated by continual IGF-II-mediated stimulation of molecules upstream of Akt/PKB rather than their lack of dephosphorylation. These findings represent a potential mechanism through which IGF-II overexpression may predispose the mammary gland to tumor development.

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Figure Legends

Figure 1. Generation and characterization of MMTV-IGF-II transgenic mice. (A) Transgenic expression cassette containing an MMTV-LTR promoter, an untranslated portion of the Ha-*ras* gene, full-length human IGF-II cDNA and a SV40 polyadenylation sequence. Numbers and arrows indicate the location of PCR primers. Primers 1 and 3 were used for RT-PCR while primers 2 and 3 were used for PCR genotyping of MMTV-IGF-II transgenics. (B) Southern analysis of IGF-II in wild type (Wt) mice and MMTV-IGF-II transgenic (Tg) lines MI1, MI12, and MI16. The IGF-II transgene (t-IGF-II) was detectable in all three transgenic lines as an 833 bp fragment while the endogenous IGF-II (e-IGF-II) gene was detected in both wild type and transgenic mice as a 1391 bp fragment. (C) RT-PCR of mammary RNA from 35-day-old MMTV-IGF-II transgenic and wild type mice using primer sets specific for IGF-II (t; primers 1 and 3) and for a control (c) transcript (IL-2). Transgene expression was restricted to the three transgenic lines while the PCR product for the control transcript was apparent in all samples. Probing with a [^{32}P]-labeled Ha-*ras* probe (transgene-specific probe) confirmed that the PCR products were the result of transgene expression. In situ hybridization using a Dig-labeled IGF-II riboprobe on sections from (D) wild type or (E) transgenic mammary tissue at d3i or a Dig-labeled Ha-*ras* riboprobe in (F) wild type or (G) transgenic mammary tissue at d3i. Both probes demonstrated elevated levels of IGF-II expression in the mammary epithelial cells of the transgenic mice compared to controls. Arrowheads indicate positive stained cells; scale bars, 50 μm.

Figure 2. Mammary IGF-II bioactivity in MMTV-IGF-II transgenics. (A) Slot blot analysis of e-IGF-II and t-IGF-II mRNA relative to 18S rRNA in involuting (o) wild type and (•) transgenic mammary tissue from days 10L to d5i. (B) Ligand blot of IGFBP levels in wild type and transgenic mammary tissue during involution. (C) Western analysis of p-IRS-1 in wild type and transgenic 10L mammary tissue. The levels of p-IRS-1 and total IRS-1 were detected using antibodies specific to either the phosphorylated form of IRS or non-phosphorylated IRS-1 and quantified by densitometry.

Figure 3. (A) Mammary gland (Mg) to body weight ratios during involution in (•) transgenic and (o) wild type mice. Values are presented as mean ± SEM; (*) $p < 0.09$, (**) $p < 0.05$. Whole mount analysis of involuting 4th-inguinal mammary glands in (B) transgenic and (C) wild type mice at d4i. Arrowheads indicate dilated lobulo alveoli; scale bars, 800 μm. Hematoxylin/eosin-stained sections of (D) transgenic and (E) wild type mice at d4i. Arrowheads indicate structurally intact lobulo-alveoli while arrows indicate collapsed lobulo-alveoli, scale bars, 200 μm. Hematoxylin/eosin-stained sections of d5i involuting mammary tissue in (F) MI16 transgenic mice and (G) MI1 double hemizygous transgenic mice. Arrowheads indicate structurally intact lobulo-alveoli, scale bars, 200 μm. (H) Quantification of the number of structurally intact lobulo alveoli in wild type and transgenic mice spanning days 2-5 of involution. Values are presented as mean ± SEM and were considered significantly different (*) when $p < 0.05$.

Figure 4. Mammary epithelial apoptosis during involution. Apoptotic epithelial cells were identified immunohistochemically in (A) transgenic and (B) wild type d3i mammary

tissue. Arrowheads indicate apoptotic cells, scale bars 50μm (C) Percentage of apoptotic epithelial cells comprising structurally intact lobulo-alveoli in (□) wild type and (■) MI1 transgenic mice. Values represent mean ± SEM of at least 3 mice at d4i and d5i and at least 2 mice at all other time points. Differences were considered significant (*) when $p < 0.05$.

Figure 5. Western analysis of wild type and transgenic mammary tissue spanning days 10L to d4i. (A) Levels of Akt/PKB as detected by the anti-phospho-Akt(Ser473) and anti-Akt antibodies in two independent sets of animals. (B) Quantification of the average levels of phosphorylated Akt/PKB normalized to the total levels of Akt/PKB in the two experiments in (o) wild type and (•) transgenic mice. Values on the Y-axis are arbitrary units. (C) Levels of PTEN protein. Lower panels represent the amido black-stained membrane to show protein loading. (D) Levels of phosphorylated Erk1 and Erk2 as well as total Erk 1 and Erk 2 protein.

Figure 6. Mammary epithelial apoptosis three days after implantation of (A) a pellet containing rIGF-II or (B) a control pellet in the 4^{th}-inguinal mammary gland of wild type mice. Arrowheads indicate apoptotic cells, scale bars 50μm. (C) Quantification of the percentage of mammary epithelial cells undergoing apoptosis surrounding either (□) a control pellet or (■) a pellet containing rIGF-II. Values represent mean ± SEM of 5 animals and these values were considered statistically significant (*) when $p < 0.05$.

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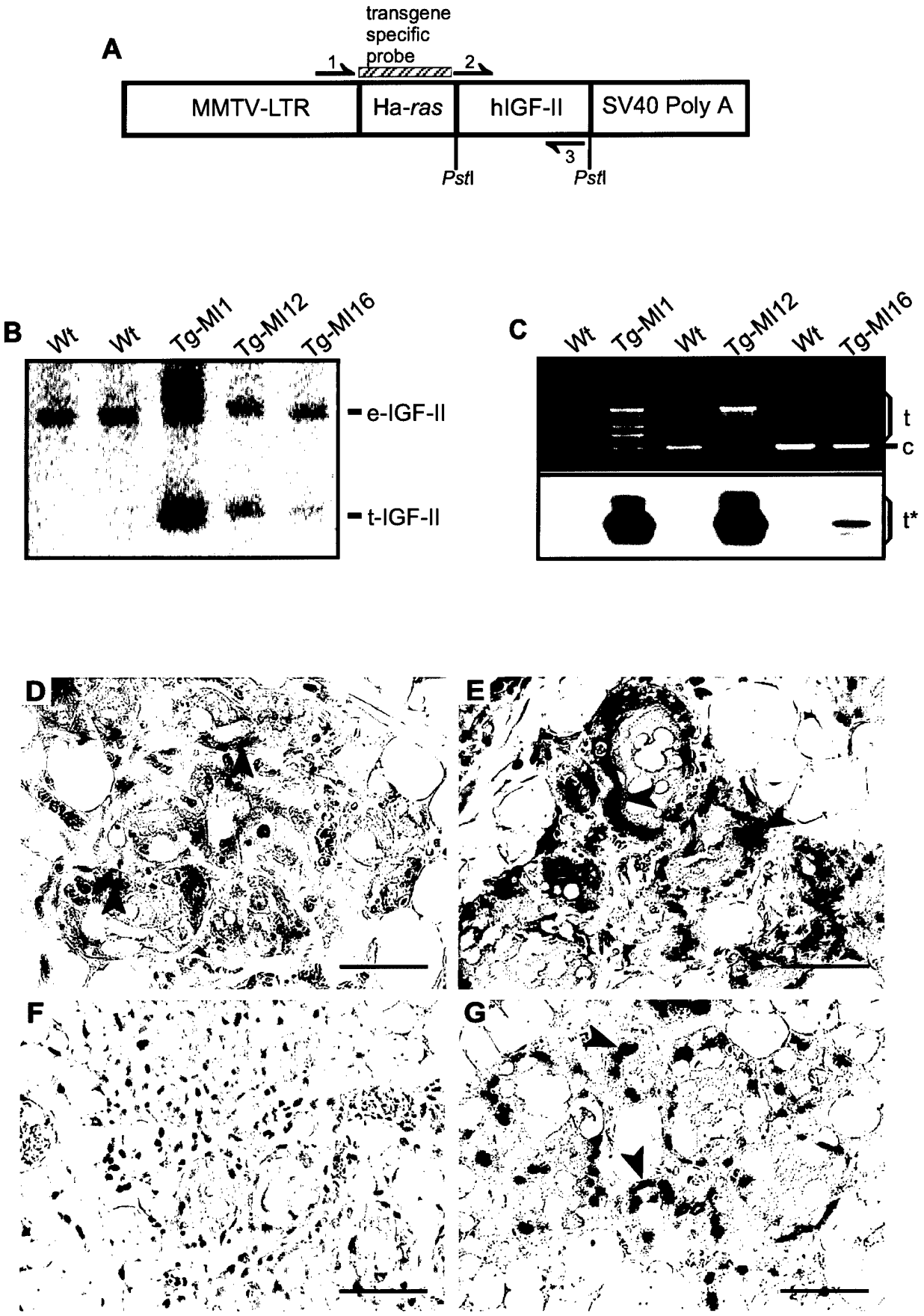
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MMTV-LTR
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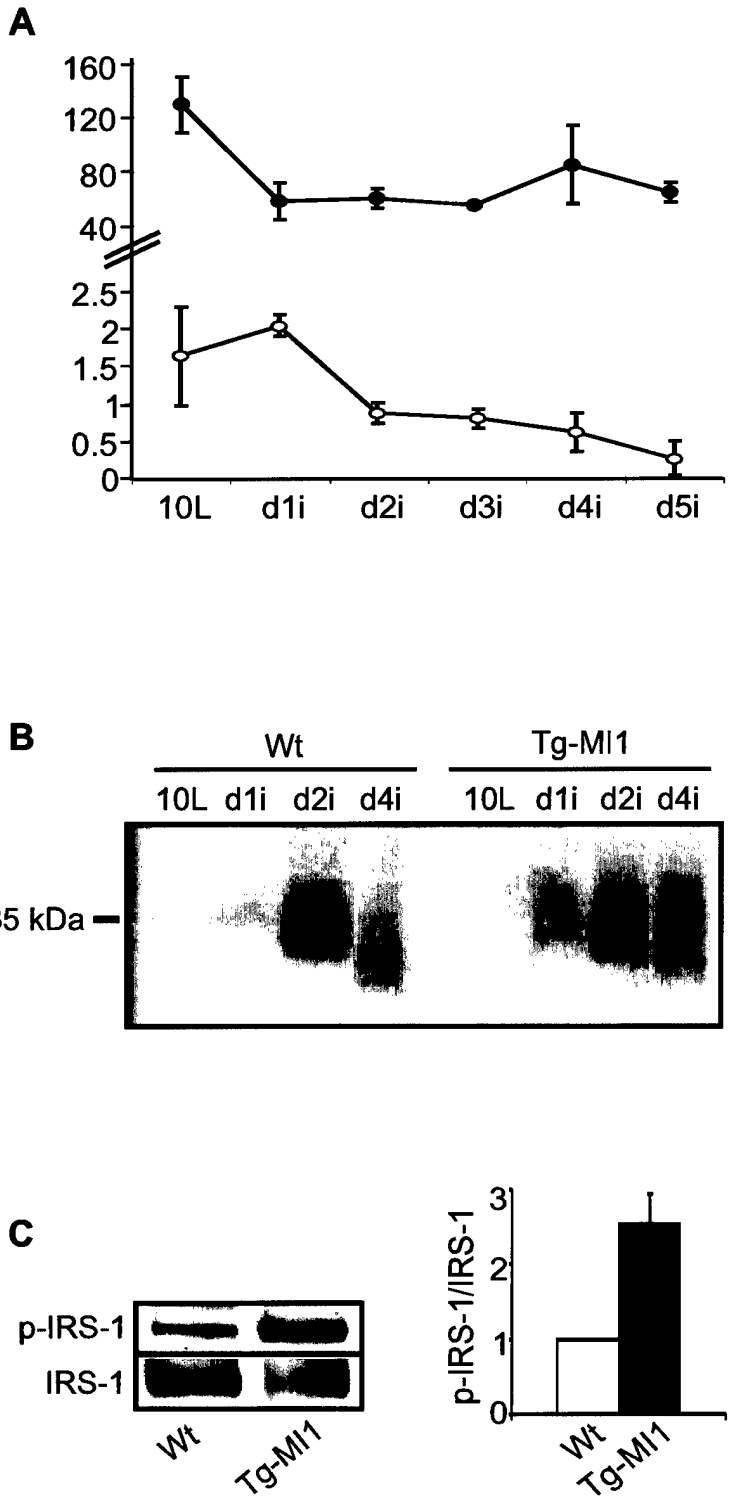
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10L d1i d2i d4i
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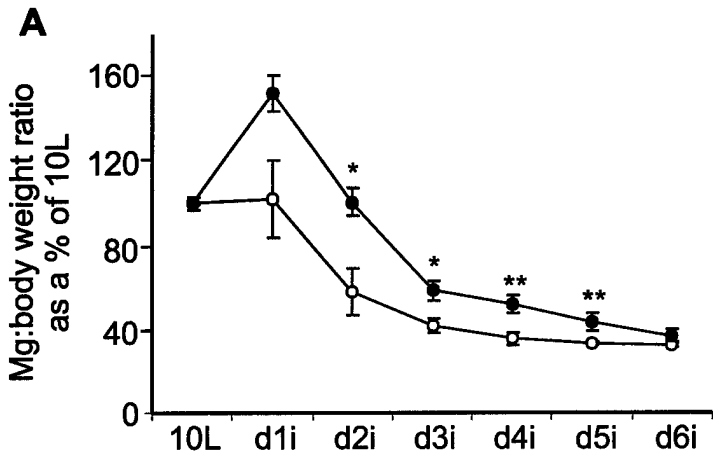
A
Mg:body weight ratio as a % of 10L
160
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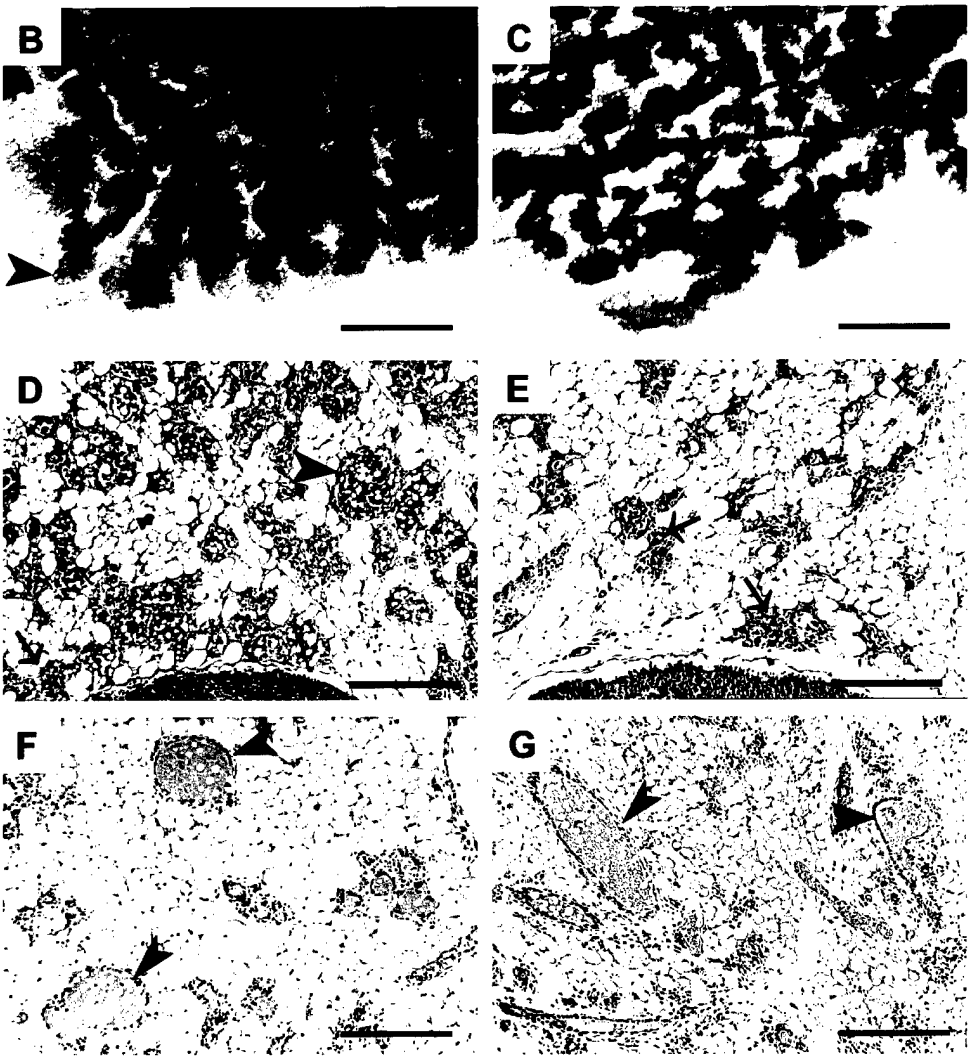
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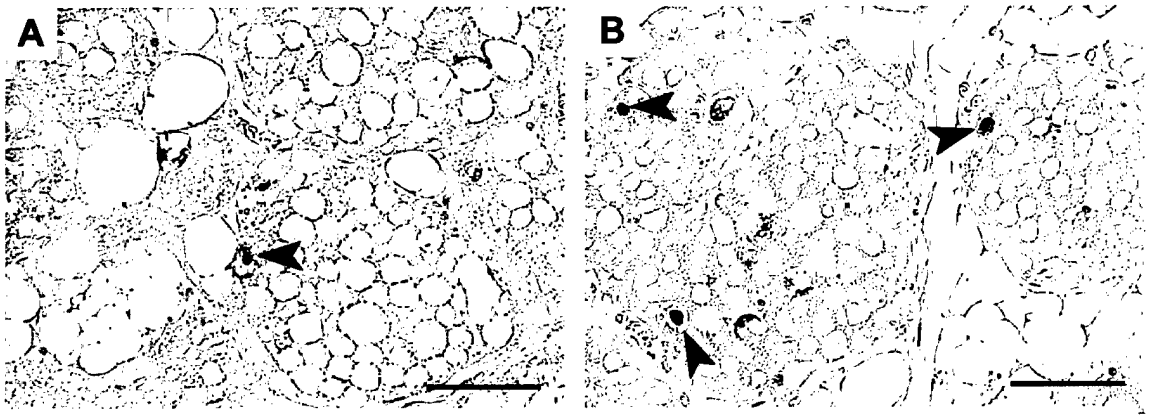
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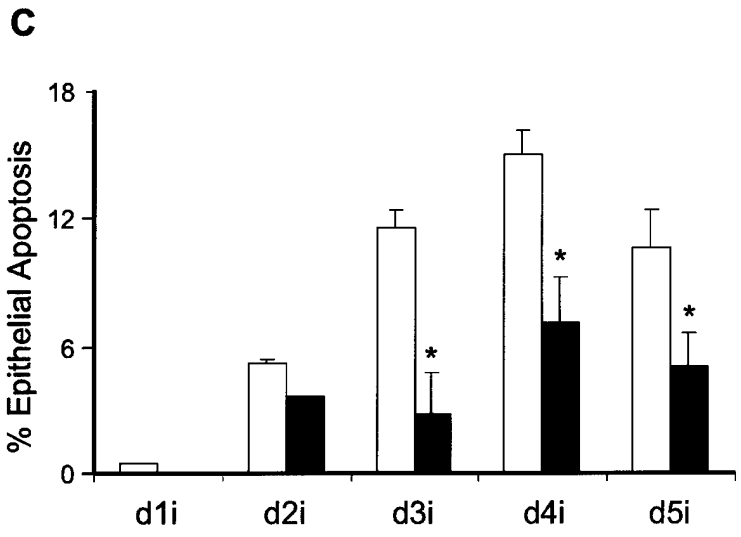
C
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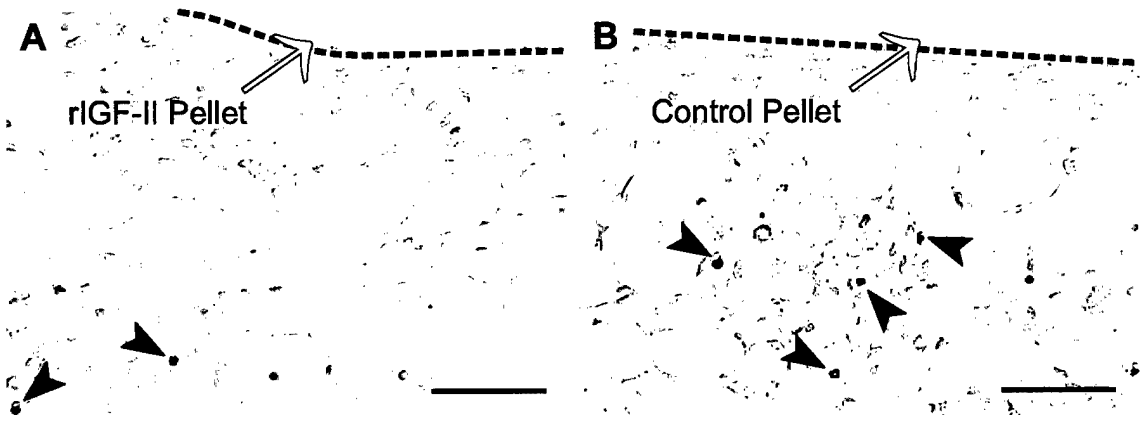
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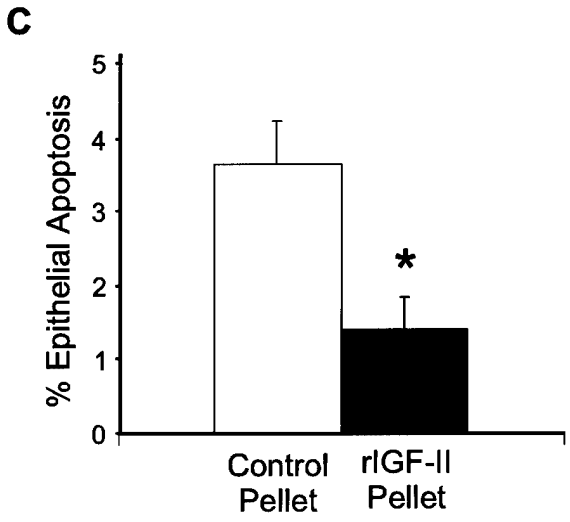
B
Control Pellet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Control Pellet
rIGF-II Pellet

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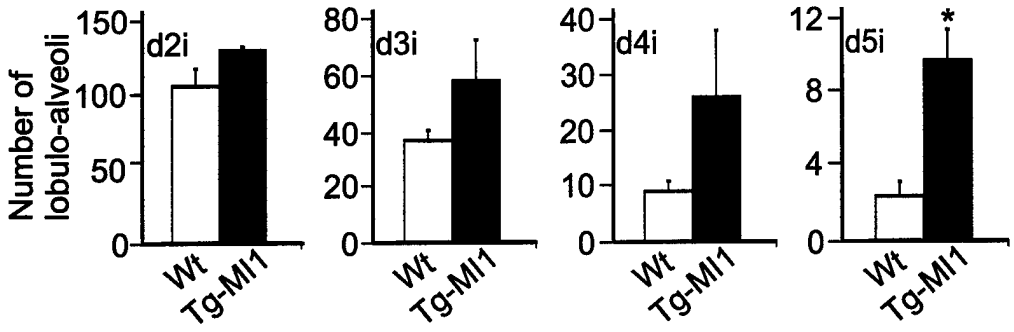
Number of lobulo-alveoli
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80
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d3i
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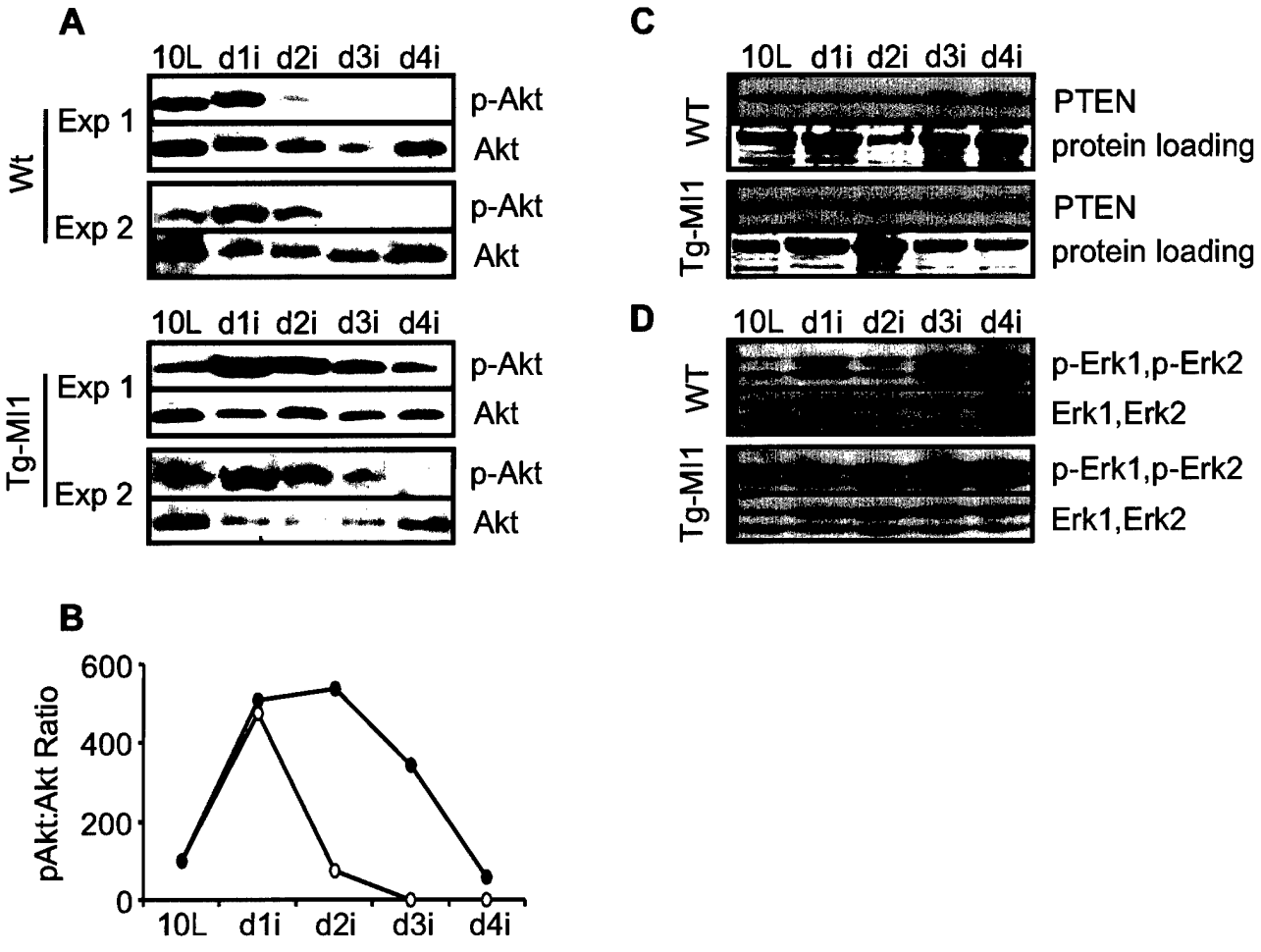
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Tg-MI1
Exp 1
p-Akt
Akt
Exp 2
p-Akt
Akt
B
pAkt:Akt Ratio
600
400
200
0
10L d1i d2i d3i d4i
C
10L d1i d2i d3i d4i
WT
PTEN
protein loading
Tg-MI1
PTEN
protein loading
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10L d1i d2i d3i d4i
WT
p-Erk1,p-Erk2
Erk1,Erk2
Tg-MI1
p-Erk1,p-Erk2
Erk1,Erk2

Delay of Mammary Involution and Epithelial Apoptosis *in vivo* by IGF-II. R.A. Moorehead, J.E. Fata, M.B. Johnson, R. Khokha. Ontario Cancer Institute/Princess Margaret Hospital, Toronto, Ontario, Canada

IGF-II overexpression is frequent in a number of human tumours including breast caner. In culture, IGF-II exerts potent mitogenic and antiapoptotic effects on breast cancer cell lines. We investigated IGF-II's ability to inhibit mammary epithelial apoptosis as well as its antiapoptotic signaling pathway *in vivo.*

Animals were manipulated genetically by generating IGF-II transgenic mice and biochemically using slow-release pellets containing IGF-II. Three independent transgenic mouse lines that overexpressed human IGF-II in their mammary glands using the mouse mammary tumour virus promoter were generated. IGF-II overexpression was confirmed at the RNA level by Northern blotting and RT-PCR, while *in situ* hybridization localized transgene-specific IGF-II message to mammary epithelial cells. In order to assess the effect of IGF-II overexpression on mammary epithelial apoptosis, transgenics and controls were subjected to forced involution initiated by litter removal at day 10 of lactation.

A delay in mammary involution was observed in transgenic mice compared to wild-type controls as evident by maintenance of lobulo-alveolar structures using whole-mount analysis. Histologically, maintenance of mammary alveoli was prolonged in transgenic mice compared to controls and there was significantly more cellularity at day 5 of involution (d5i) in the mammary glands of the transgenics. Immunohistochemical detection of apoptotic cells indicated that epithelial apoptosis was reduced in transgenic mice at d3i, d4i, and d5i compared to controls. An extended activation of the antiapoptotic protein, Akt/PKB, in transgenic mice was, at least in part, responsible for the decrease in apoptosis. Using a phospho-specific Akt/PKB antibody, phosphorylated Akt/PKB could be detected until d4i in transgenic mice but only until d2i in wild-type mice. An alternative, biochemical approach was used to further test the antiapoptotic effect of IGF-II on mammary epithelium, through implanting control pellets or pellets with recombinant IGF-II in the mammary glands of wild-type mice at d2i. Mammary epithelial apoptosis around IGF-II pellets was decreased compared to the contralateral mammary gland bearing control pellets when examined 3 days later (d5i).

Our data indicate that overexpression of IGF-II delays mammary epithelial apoptosis during involution and that IGF-II may contribute to mammary tumourigenesis through inhibiting epithelial apoptosis.